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stereochemical correlations
terpenes 3. steroids
drugs 5. retinene isomers
enzymes
nucleotide metabolism
enzyme action

BIOCHEMICAL SOCIETY SYMPOSIA

NO. 19

STERIC ASPECTS OF THE CHEMISTRY AND BIOCHEMISTRY OF NATURAL PRODUCTS

STERIC ASPECTS OF THE CHEMISTRY AND BIOCHEMISTRY OF NATURAL PRODUCTS

Biochemical Society Symposium No. 19
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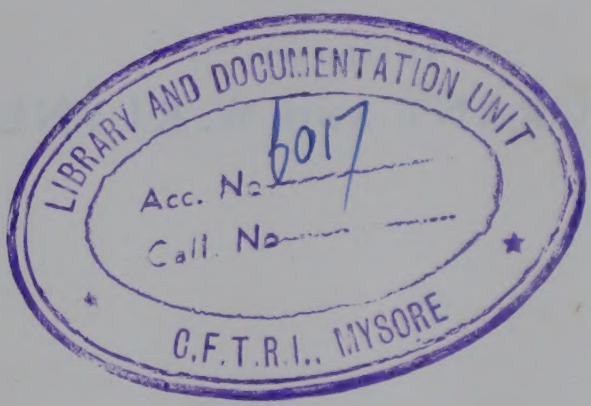

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NOTES

(1) It may be appropriate to draw attention to a report, recently published, of a meeting held in March 1959 at the Ciba Foundation, at which the steric specificities of microbiological reactions were considered. Several of the papers in this report complement and extend those given in the present symposium (Ciba Foundation Study Group, 1959, no. 2, *Steric Course of Microbiological Reactions*. London: J. and A. Churchill Ltd.).

(2) *Stereochemical nomenclature.* Several different systems have been and are used to indicate the configurations of stereoisomers.

(*a*) The small capital letters D and L are used in the carbohydrate, amino acid and related fields to indicate the relationship of a compound to glyceraldehyde or to serine. The use of these symbols is no doubt familiar to most readers; convenient references are *J. chem. Soc.* (1952), p. 5108 (editorial report; carbohydrates); *Biochem. J.* (1948), **42**, 1; (1952), **52**, 1 (amino acids); Mitchell, A. D., *British Chemical Nomenclature* (1948; London: Arnold); Cahn, R. S., *An Introduction to Chemical Nomenclature* (1959; London: Butterworths Scientific Publications), a valuable, short, general treatment of nomenclature problems.

The symbols D and L should *not* be used except for compounds which have been correlated with glyceraldehyde or serine.

(*b*) The italic capital letters *R* and *S* may be used for stereoisomers of *all* kinds in accordance with the proposals of Cahn, R. S., Ingold, C. K. & Prelog, V. [*Experientia* (1956), **12**, 81]. This system, which is based on the use of a Sequence Rule, is independent of projection formulae and is applicable to all types of structures, including branched-chain compounds, spirans, allenes, etc. Although highly logical, this system may appear at first sight difficult to follow; its use—alongside 'local' nomenclature for special fields such as the steroids—is steadily growing because of its universal applicability.

(*c*) The symbols (+), (−) or (±) indicate the actual sign of rotation of a compound, without reference to its stereochemistry. The words (*dextro*), (*laevo*) or racemic may be used as alternatives.

(*d*) The lower-case italic letters *d*, *l* and *dl* have been used in the past to indicate sometimes steric relationships, and sometimes sign of rotation; they should *not* be used in either sense.

(*e*) Italic capital letters *D* and *L* are sometimes used (although not in any of the papers here) to indicate the configuration of a particular centre in an acyclic compound. Although a 'trivial' system this is often convenient, e.g. for branched-chain fatty acids. [See Linstead, R. P., Lunt, J. C. & Weedon, B. C. L. (1950), *J. chem. Soc.* p. 3333; Ställberg-

Stenhagen, S. (1949), *Ark. Kemi*, **1**, 187; Klyne, W. (1951), *Chem. & Ind.* p. 1022.]

(*f*) The Greek letters α and β used in steroids to indicate respectively groups below and above the plane of the ring system [cf. IUPAC, *Nomenclature of Organic Chemistry* (1957), London: Butterworths Scientific Publications] are now applied in similar fashion to other polycyclic systems, both carbocyclic (diterpenes, triterpenes) and heterocyclic (yohimbine and tropane alkaloids).

INTRODUCTORY REMARKS

By A. NEUBERGER

Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

Stereochemistry deals with the configuration of molecules in the solid, liquid or gaseous state and with the effect of molecular configuration on physical and chemical properties. The special interest which the biochemist takes in configuration or spatial arrangement of molecules arises in the first place from the fact that the majority of the natural products with which he deals are asymmetric, i.e. these compounds can exist in two or more configurations or states of spatial arrangement which may or may not differ in internal energy or other properties. An understanding of the stereochemistry of compounds of biological interest is of importance in the elucidation of biogenesis, in the understanding of structures of complex molecules and particularly in the interpretation of the mechanisms of action of enzymes.

Stereochemistry began with the observation of Biot in 1815, showing that certain organic liquids such as oil of turpentine rotate the plane of polarized light. But it was Pasteur who carried out the first resolution of a simple compound—sodium ammonium tartrate. In a later paper Pasteur showed that asparagine and aspartic acid rotate polarized light to the left and right respectively. He also found that the addition of hydrochloric acid or nitric acid changed the small negative rotation of free asparagine to a positive value, whilst addition of alkali increased slightly the laevorotation. This observation of Pasteur was later extended by Clough, Levene, Lutz & Jirgensons to most other α-amino acids and, together with other evidence provided by the work of Emil Fischer, of Karrer and of Levene, led to the conclusion that all amino acids occurring in proteins are configuratively related and have in fact the L-configuration. It has been reported by Kögl that tumour proteins may contain D-glutamic acid, but later work has not given much support to this idea and we may assume for the time being that protein synthesis is absolutely stereospecific as far as the configuration of the α-carbon atom of the constituent amino acids is concerned. D-Amino acids are of course fairly widespread in Nature. Thus Wintersteiner *et al.* (1913) isolated DL-alanine from the mushroom *Boletus edulis* and Ackermann & Mohr (1937) obtained the picrate of D-ornithine from the liver of the fish *Acanthias vulgaris*. The occurrence of D-amino acids in fungi and micro-organisms is fairly common. Thus the glutamic acid residue in the polyglutamic acid occurring in the capsular substance of the anthrax

bacillus has the D-configuration. Similarly, the penicillamine moiety of penicillin was shown to have D-configuration by conversion into D-valine, and this deduction was confirmed by the X-ray studies of Crowfoot. In this connexion the studies of Arnstein and Stevens have clearly indicated that L-cysteine and L-valine are precursors of penicillin and it has been suggested that L-cysteinyl-L-valine is an intermediate, making it likely that the D-configuration of the penicillamine residue in penicillin arises during the ring closure of the fused β-lactam–thiazolidine structure. In other words, penicillamine is not an intermediate in the biosynthesis and is almost an artifact. D-Amino acids have been found in actinomycin, bacitracin, polymyxin, gramicidin and tyrocidin, and Arnstein has suggested that the cell might make both D- and L-peptides, but that the latter only are degraded.

The classical period of stereochemistry was distinguished by the elucidation of the structures and configurations of the sugars so brilliantly carried out by Fischer and by the contributions of Perkin, Mills and Pope in this country. After the First World War, interest in stereochemistry lessened somewhat, but in the last ten or fifteen years both chemists and biochemists have paid increasing attention to this field of science. In the first place, X-ray crystallography and other methods focused attention again on the shapes of molecules. Secondly, as organic chemists took up seriously the problems of kinetics and mechanisms of organic reactions it became clear that an understanding of stereochemistry was of vital importance in the interpretation of results and a similar situation has developed with respect to enzymology. It is therefore most appropriate that The Biochemical Society should organize a Symposium on Stereochemistry. It may be difficult to single out contributions made in the last decade which are of particular importance, but I should like to name three which appear to me to be of general significance. As the first example I wish to cite the generalization that any bimolecular aliphatic substitution, particularly nucleophilic substitution, is associated with a Walden inversion. As my second example I should like to take the work of Hassel and others on the effect of conformation on the properties and reactivities of alicyclic rings. This had led to a large amount of fruitful work in the field of steroids and sugars, and is of the most direct interest to the biochemist. Finally, I should like to cite the work of Ogston, who has pointed out that an enzyme could distinguish between the two *a* groups of a compound C(*aabc*). This idea, which now seems obvious to us, but was surprising when it was first put forward, has greatly influenced our thinking and has been of considerable importance in helping us to understand various enzymic and metabolic reactions.

STEREOCHEMICAL CORRELATIONS

By W. KLYNE

Postgraduate Medical School, London, W. 12

Stereochemical correlation means the study of the relative and absolute configuration of asymmetric compounds. Since most biologically active compounds are asymmetric, knowledge of their configurations is essential both for the structural organic chemist and the biochemist. The determination of the structure of an asymmetrical natural product should not be considered as complete until its absolute configuration is known.

Stereospecificity is characteristic of many (perhaps all) enzymes, and therefore steric relationships are important in biochemical work at all levels—from studies of isolated pure enzymes to studies on the whole organism—and of all types, e.g. work on metabolism, biogenesis, drug action.

Hopkins (1933) stated the essential aim of biochemistry as 'an adequate and acceptable description of molecular dynamics in cells and tissues'. To be adequate, any such description must be three-dimensional, and therefore no further justification is necessary for emphasizing the steric aspects of biochemistry.

Much important work is in progress on the shape and structure of proteins, nucleic acids and polysaccharides. (For a brilliant outline see Perutz, 1958.) A full understanding of biochemical processes, including enzyme–substrate interactions, will require the extension of these structural studies to finer points of detail; this in turn will require a full knowledge of the relative stereochemistry of the smaller molecules, which act as building-blocks for the organic macromolecules and as substrates. This has been pointed out by Theorell (1957) in a Nobel Prize address, and, from the organic-chemical side, two leading workers have suggested the preparation of 'imitation enzymes' as one of the next major goals for synthetic studies (Todd, 1956; Woodward, 1956).

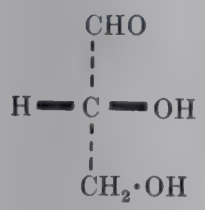
Until a few years ago, all configurations were designated relative to an arbitrary standard (glyceraldehyde or serine). It is now known, thanks to the X-ray work of Bijvoet, Peerdeman & van Bommel (1951) (see p. 14) that, by a fortunate chance, the Fischer convention for glyceraldehyde represents its true absolute configuration.

The methods for correlating configurations have been considered in detail elsewhere in a full review (Mills & Klyne, 1954). For a long time too little attention was paid, either by organic chemists or by biochemists, to problems of correlation, but over the last five years

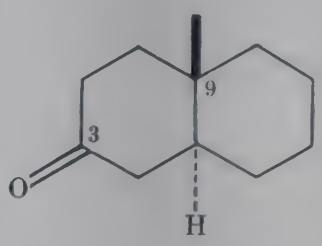
much important work has appeared; it is gratifying to find how often evidence of different types provides cross-checks, so that the whole body of correlations is now firmly established. Examples taken from different fields of organic chemistry, and chiefly from recent literature, will be used here to illustrate the principal methods.

Formulae and conventions. All formulae show known absolute configurations. Acyclic formulae follow the usual Fischer convention (now known to be correct in absolute terms), e.g. (I) for D-glyceraldehyde. Where it is convenient to do so, bonds above and below the plane of the paper are shown by thick and dashed lines respectively.

Cyclic formulae follow steroid conventions (also known to be correct in absolute terms); e.g. (II) for the methyldecalone corresponding to the A and B rings of 5α-cholestan-3-one. Bonds above and below the plane of the paper are shown by thick and dashed lines respectively.



(I)
D-Glyceraldehyde



(II)
trans-9-Methyl-3-decalone

The symbol *E* is used for 'enantiomer of'; thus, for example (*E*, IX), means 'enantiomer of formula IX'.

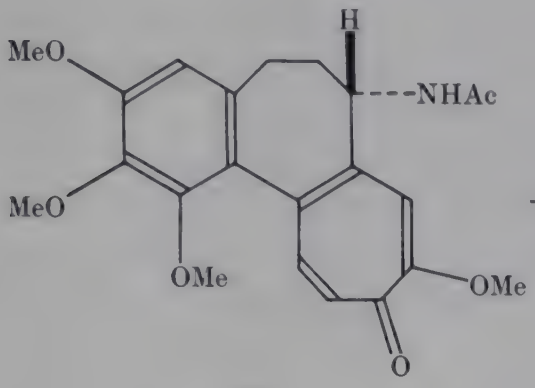
Types of evidence used for correlations. These may be classified as follows: (1) Chemical reactions, without displacement at the asymmetric centre concerned. (2) Chemical reactions, with displacement at the asymmetric centre concerned. (3) Asymmetric synthesis. (4) Formation of quasi-racemates. (5) X-ray crystallographic studies: (*a*) of absolute configuration; (*b*) of relative configuration. (6) Optical-rotation evidence: (*a*) monochromatic rotations; (*b*) rotatory dispersion. (7) Enzymic studies.

CHEMICAL REACTIONS WITHOUT DISPLACEMENT

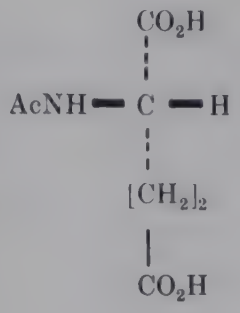
These are reactions in which no atom directly attached to the asymmetric centre under consideration is displaced; i.e. in which no bond of the asymmetric atom is broken. These reactions provide the safest and most certain correlations; they may be syntheses or degradations.

Good examples of the degradative method are provided by correlations in which large fragments of a complex molecule are oxidized

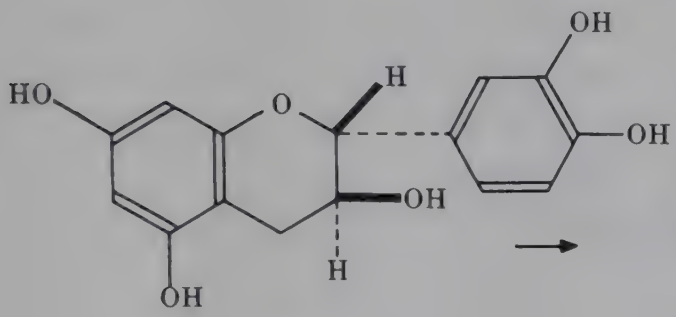
away, and a small recognizable fragment of known stereochemistry is left. This method has been much used in recent years by the Zürich school (Hardegger, Jeger and their colleagues). Examples are as follows: (*a*) Colchicine (III) on ozonolysis yields *N*-acetyl-L-glutamic



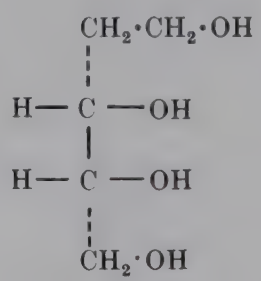
(III)
Colchicine



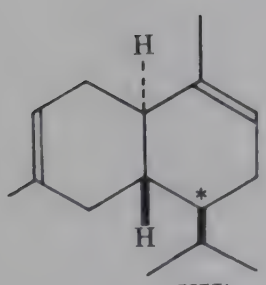
(IV)
N-Acetyl-L-glutamic acid



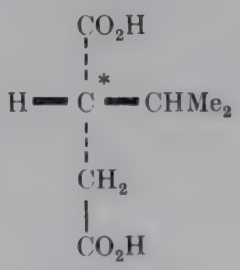
(V)
(+)-Catechin



(VI)
2-Deoxy-D-adonitol

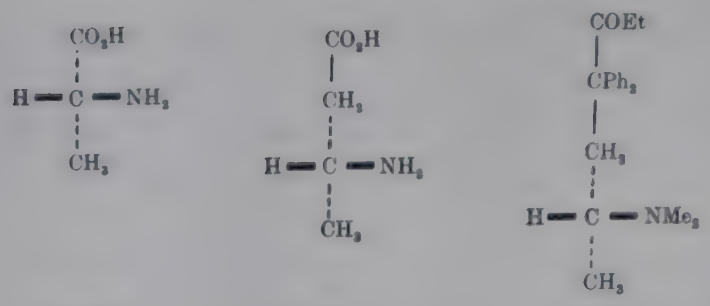


(VII)
β-Cadinene



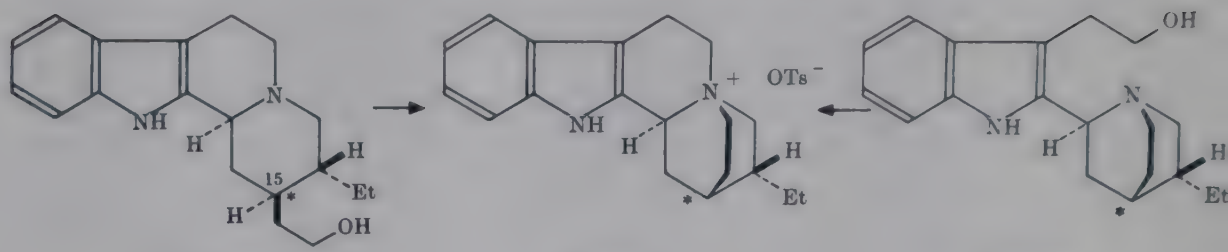
(VIII)
*α-iso*Propylsuccinic acid

acid (IV), thus establishing the stereochemistry of the only asymmetric centre in (III) (Corrodi & Hardegger, 1955). (*b*) The flavans, represented by (+)-catechin (V). This on ozonolysis and subsequent reduction yields 2-deoxy-D-adonitol (VI), thus establishing the absolute configuration of both asymmetric centres in (V) (Hardegger, Gempeler & Zust, 1957; see also Kulkarni & Joshi, 1957; Birch, Clark-Lewis &



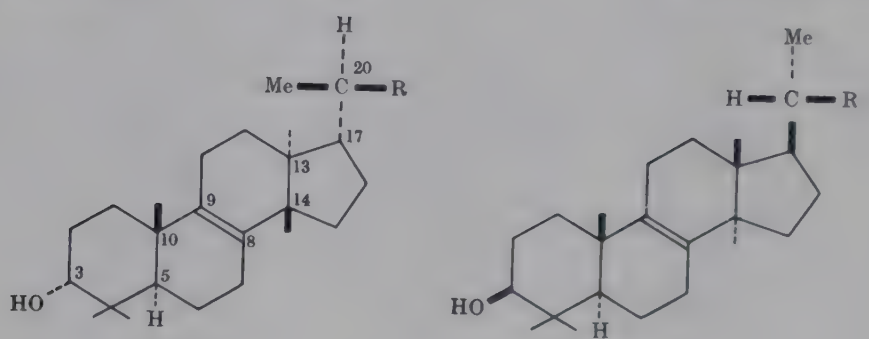
(IX) (X) (XI)

D-Alanine D-β-Aminobutyric acid (−)-Methadone



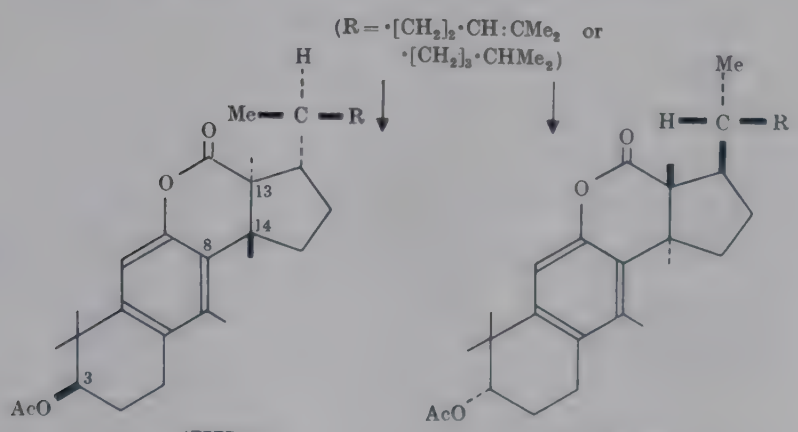
(XII) (XIII) (XIV)

Dihydrocorynantheol Dihydrocinchonamine



(XV) (XVI)

Tirucallol Lanosterol



(XVII) (*E*, XVII)

Enantiomeric acetoxyphenol-lactones

Robertson, 1957 (asymmetric synthesis); Freudenberg, 1956 (rotations)). (*c*) The sesquiterpene, β-cadinene (VII), on ozonolysis yields α-*iso*-propylsuccinic acid (VIII) of known configuration (Sýkora, Herout & Šorm, 1958).

The alkylsuccinic acids are important reference standards for all branched-chain structures, e.g. the macrolide antibiotic methymycin

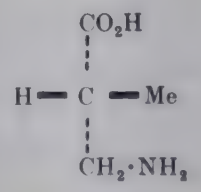
has been degraded to L-(−)-methylsuccinic acid (Djerassi & Halpern, 1957).

A synthetic example is provided by the later stages of the preparation of (−)-methadone (XI) from D-alanine (IX) (Beckett & Harper, 1957). The early stages of this process are used as an example of correlation using a displacement reaction (see p. 8).

Another ingenious example of synthesis, using this term in the sense of forming carbon–carbon bonds, is provided by the work of Wenkert & Bringi (1959), who converted dihydrocorynantheol (XII), a representative of the yohimbine–corynantheine–ajmalicine group, and dihydrocinchonamine (XIV), a representative of the cinchona alkaloids, whose configuration was already known, into a common quaternary salt (XIII), thus correlating their stereochemistry. The common stereochemical feature of all these structures is the configuration of C-15 (yohimbine numbering; marked with an asterisk). This work confirmed the previous allotment of absolute configuration to yohimbine on rotational evidence by Klyne (1953*a*), Bose, Chatterjee & Iyer (1956) and Djerassi, Riniker & Riniker (1956; rotatory dispersion).

A process which is sometimes convenient to correlate two structures, which are largely but not entirely enantiomeric in nature, is to destroy the asymmetry at some centres and invert the stereochemistry at others so that two true enantiomers are obtained. An excellent illustration is the correlation of tirucallol (XV) with the steroid-like tetracyclic triterpene lanosterol (XVI), as shown in the accompanying formulae, through the enantiomeric acetoxyphenol-lactones (XVII and *E*,XVII) (Menard *et al.* 1955). This demonstrates that the compounds of the elemadienolic acid–euphorbol–tirucallel series are epimeric with lanosterol at all four positions 13, 14, 17 and 20. The correlation depends on a sequence of reactions applied in the lanostane series by Barnes, Barton, Fawcett & Thomas (1952), in which the asymmetry at C-5 and C-10 is destroyed in the aromatization of ring B. This enantiomer-forming technique was also applied by Wenkert & Bringi (1959) in their work on the yohimbé alkaloids.

A further synthetic correlation is that of Balenović & Bregant (1957), who linked the urinary β-amino acid, (−)-α-methyl-β-alanine (XVIII) (Crumpler, Dent, Harris & Westall, 1951), with (−)-2-methylbutanol.



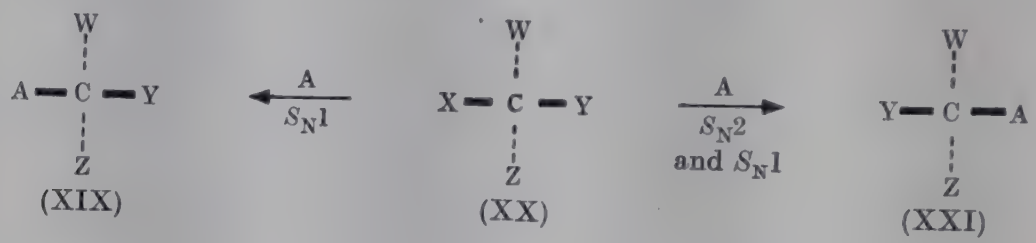
(XVIII)

A combination of degradative and synthetic reactions (also supported by rotational evidence) has given the absolute configuration of the important group of lignans (Carnmalm, 1956; Schrecker & Hartwell, 1957).

CHEMICAL REACTIONS WITH DISPLACEMENT

These are reactions in which displacement of one group attached to the asymmetric centre takes place. If they are to be of any value for correlations the reaction must necessarily be one whose steric consequences (retention or inversion of configuration) are known.

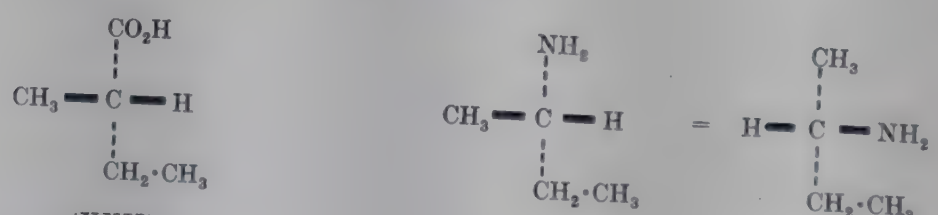
The classical studies of Ingold, Hughes and their colleagues at University College London on nucleophilic substitution have shown that unimolecular reactions of this type (S_N1) generally occur with racemization, whilst the bimolecular reactions (S_N2) generally occur with inversion of configuration. This may be represented diagrammatically as follows:



(XIX) (XX) (XXI)

For a summary see Ingold (1953).

Few examples are known outside the work of the Ingold school in which kinetic measurements have been used to indicate the nature, and hence the stereochemistry, of the displacement reaction. There are, however, many cases where reactions, whose stereochemistry is well known from other studies, have been used to deduce by analogy otherwise unknown absolute configurations.



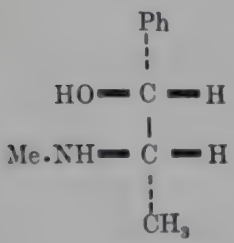
(XXII) (XXIII)

Arguments by analogy involving retention of configuration are represented by the following. Balenović, Cerar & Fuks (1952) transformed L-alanine (*E*,IX) into β-aminobutyric acid (*E*,X) by Wolff rearrangement of the diazo ketone corresponding to (*E*,IX); this rearrangement takes place with retention of configuration at the asymmetric carbon atom (for references see Ingold, 1953, p. 502): therefore the β-amino acid has the same configuration as the original α-amino acid, as shown.

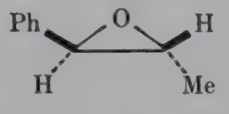
Kjaer & Hansen (1958) degraded (+)-2-methylbutyric acid (XXII) to (+)-2-aminobutane (XXIII) by the Curtius rearrangement, which

is known to occur with retention. This provides a cross-check between series which had already been correlated.

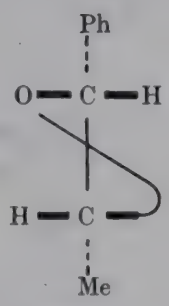
An example where the analogy involves inversion of configuration is the work of Witkop & Foltz (1957) on ephedrine (XXIV). It has long been known from degradative evidence that the configuration of the α-centre in ephedrine is as shown in (XXIV), but the configuration at the β-centre was unknown. Treatment of (XXIV) (as its quaternary salt) with silver oxide yielded a β-methylstyrene epoxide which was shown, by infrared comparison with the racemic product, to be the *trans*-isomer (XXV). (XXV) may be written as (XXVA) to show its derivation from a phenylpropane derivative such as (XXIV). It is known that displacement reactions involving the formation or the breaking of a three-membered heterocyclic ring generally occur with inversion at the point where a C–X bond is broken; therefore the configuration of (XXIV) at C-β must have been inverted to give (XXV), as shown.



(XXIV)
Ephedrine



(XXV)



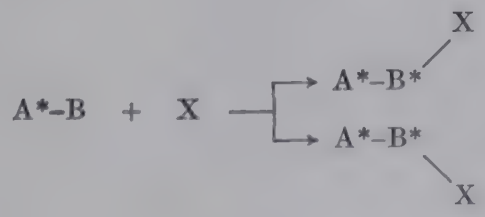
(XXV A)

β-Methylstyrene epoxide

This work is paralleled by similar mechanistic studies of Sicher & Pánková (1955) and confirmed by the X-ray work of Phillips (1954).

ASYMMETRIC SYNTHESIS

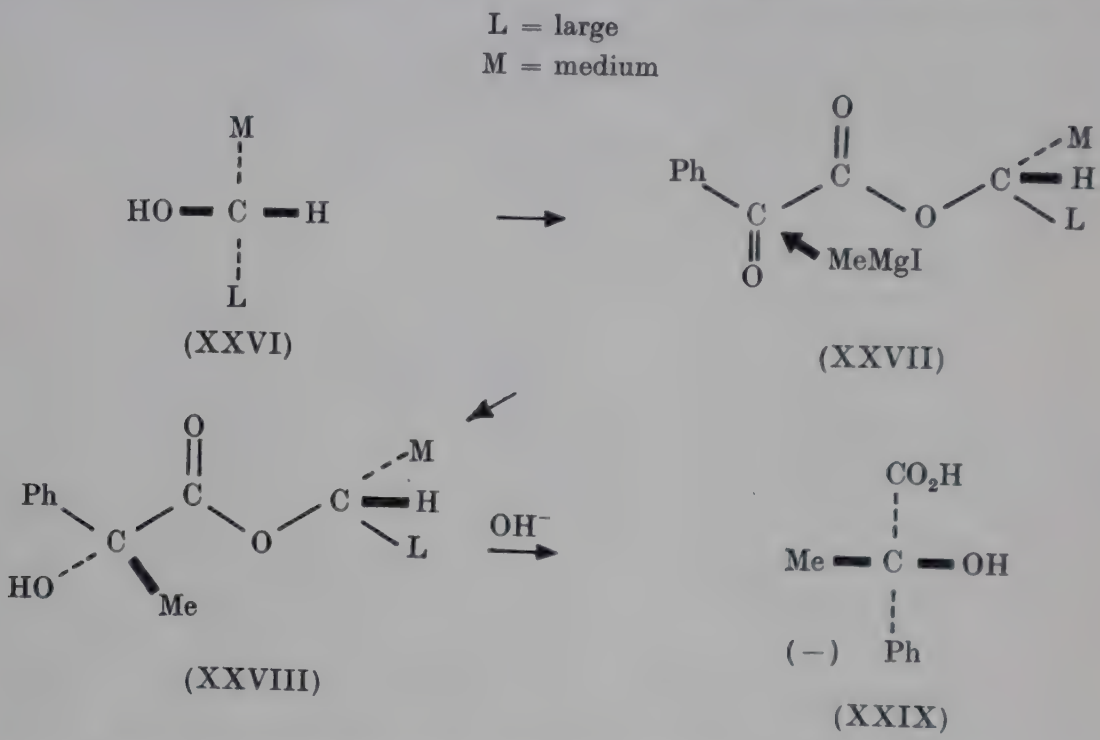
The process of asymmetric synthesis may be represented by the following generalized scheme. Consider a compound A*–B, one part of which A* contains one or more asymmetric centres, whilst the other part is symmetrical. If now a reagent X reacts with B in such a way as to make this asymmetric, two diastereoisomers (ABX) may be produced.



(1)

In general the two products will be formed in different quantities. Much early work on reactions of this type was carried out by Mackenzie and his co-workers.

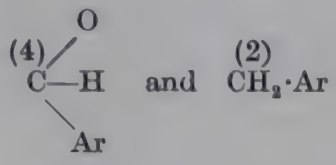
The use of such reactions to determine the configuration of the asymmetric fragment A* is relatively recent. Several different treatments have appeared within the last ten years; all depend essentially on (*a*) the very reasonable assumption that the reagent X approaches B from the least hindered side, and (*b*) the less simple matter of deciding which is the least hindered side; this often involves difficult assumptions regarding preferred conformations about single bonds. The two most extensive studies in this field are those of Prelog (1953, 1956)



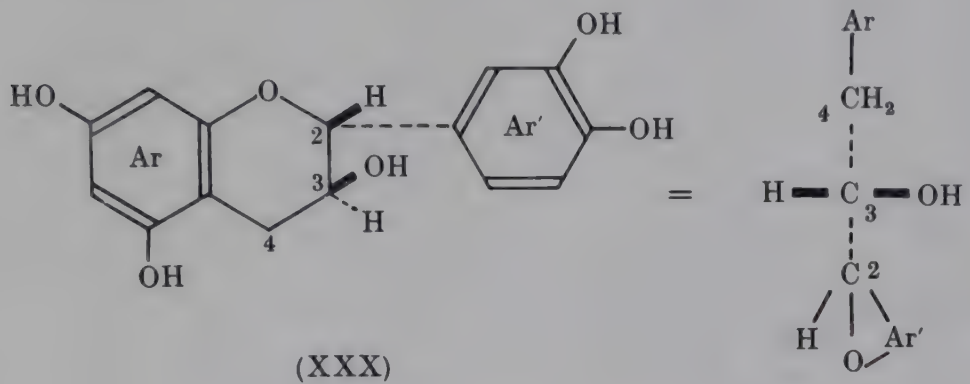
and of Cram & Abd Elhafez (1952). Prelog's method consists in taking an asymmetric alcohol, LMCH·OH [XXVI, which represents A* in equation (1)], and making its phenylglyoxylate (XXVII) LMCH·O·CO·CO·C_6H_5 (= A*–B); this is then allowed to react with a Grignard reagent (R·Mg·X). The product is a mixture of the two diastereoisomeric atrolactic esters (XXVIII) of the original alcohol. Hydrolysis yields atrolactic acid, in which one or other enantiomer (XXIX) predominates.

Prelog made a number of reasonable assumptions which permitted a correlation between the sign of rotation of the atrolactic acid produced and the configuration of the alcohol LMCH·OH. The value of the method is very greatly supported by the fact that the application of these ideas to about forty-five examples studied by Mackenzie, in which the absolute configuration of A* was already known, gave independently the correct configuration for A*.

This phenylglyoxylate method was used by Birch *et al.* (1957) to determine the absolute configuration of (+)-catechin (XXX), which provides a reference standard for the important group of the flavans. In (XXX) the groups



are considered as the large and medium groups (L) (M) attached to the asymmetric centre C-3. This allotment of configuration was confirmed by simultaneous degradative work (see p. 5).



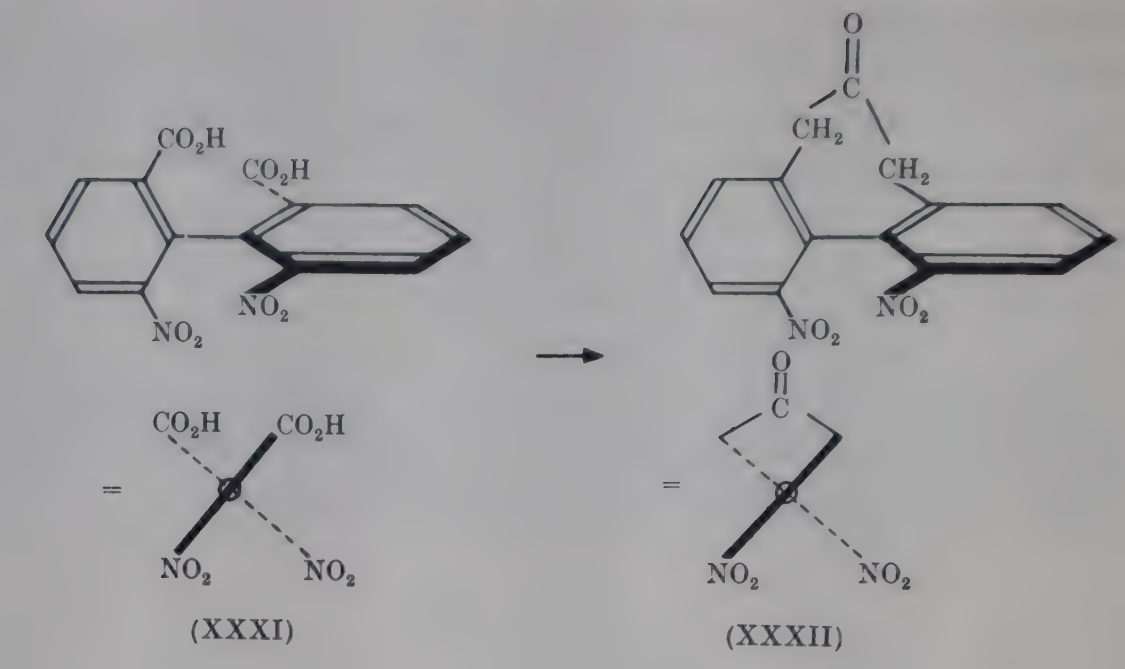
(XXX)
(+)-Catechin

Another important application of asymmetric synthesis is that of Mislow and his colleagues (Newman, Rutkin & Mislow, 1958; and four succeeding papers; see also Berson & Greenbaum, 1957), to a ketone (XXXII) related to the diphenic acids (XXXI). The preferential reduction of this ketone with (+)-pinacolyl alcohol and aluminium *tert.*-butoxide occurs as shown in formulae (XXXII–XXXIII) (p. 12).

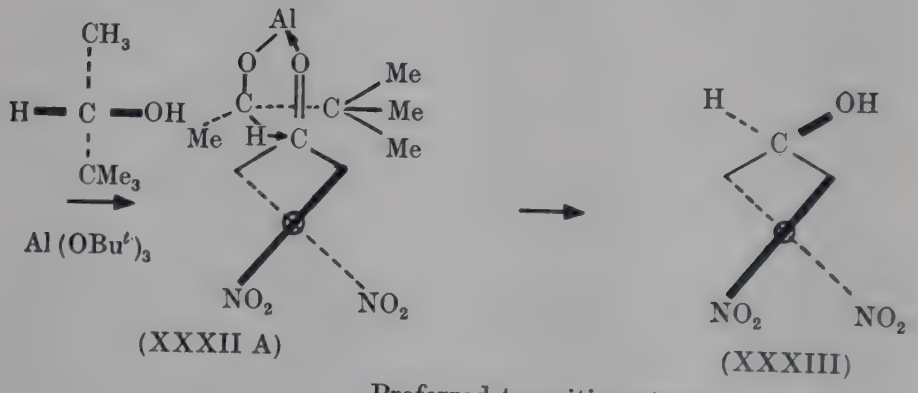
QUASI-RACEMIC COMPOUNDS

If two enantiomers (+A) and (−A) (XXXIV, XXXV) are mixed in varying proportions and a melting point–composition diagram is drawn, three types of curve may be obtained (see Fig. 1). The very characteristic type *C* with a *maximum* melting point at 50% (+A): 50% (−A) indicates the formation of a racemic compound.

If now two compounds are taken which are nearly but not quite enantiomers (+A) and (−A′) (XXXIV, XXXVI), and a similar melting point–composition diagram is made, three possibilities again are found (Fig. 2). In some cases if the compounds are sufficiently similar (except for their nearly enantiomeric nature) they may form a 1:1 molecular compound, which is called a quasi-racemic compound,



(XXXI) (XXXII)



(XXXII A) (XXXIII)

Preferred transition state.

The transition state for the reaction between (*E*, XXXII) and (+)-pinacolyl alcohol is not favoured; therefore when the reaction shown above is applied to the racemic mixture (XXXII + *E*, XXXII), it gives an excess of (XXXIII) over (*E*, XXXIII) in the product.

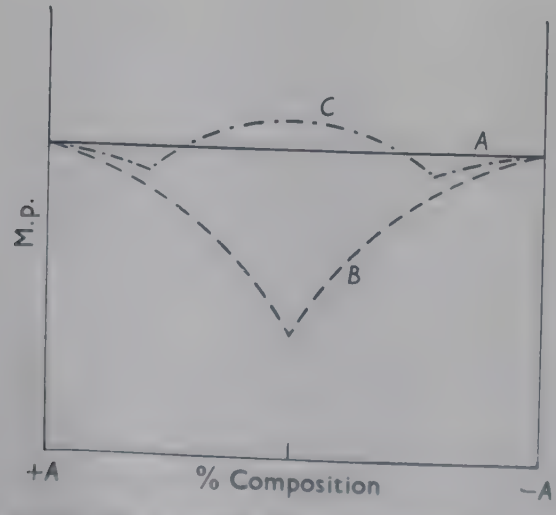
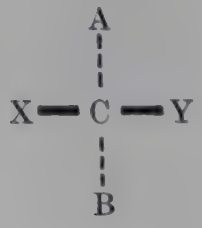


Fig. 1. Melting point–composition curves for true enantiomers (XXXIV and XXXV). *A*, solid solutions; *B*, single eutectic; *C*, racemate formation.

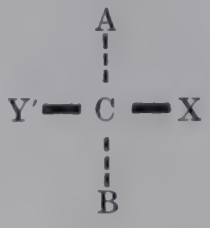
and gives a non-symmetrical curve with a maximum (curve *F* of Fig. 2).

This method of correlation was developed by Fredga in Uppsala (for reviews see Fredga, 1944, 1960) and has been used extensively by his school. A classic and simple example of great importance is the corre-



(XXXIV)
(+A)

(XXXV)
(−A)



(XXXVI)
(−A′)

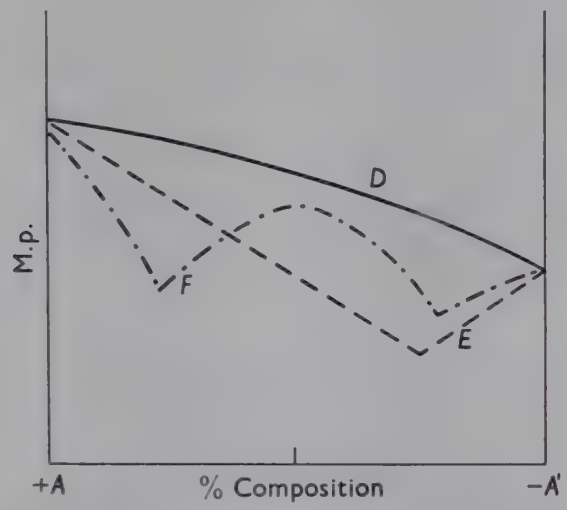
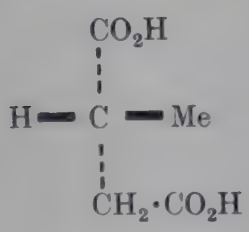
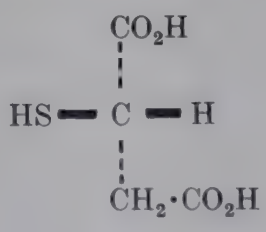


Fig. 2. Melting point–composition curves for 'quasi'-enantiomers (XXXIV and XXXVI). *D*, solid solutions; *E*, single eutectic; *F*, quasi-racemate formation.



(XXXVII)
(+)-Methylsuccinic acid

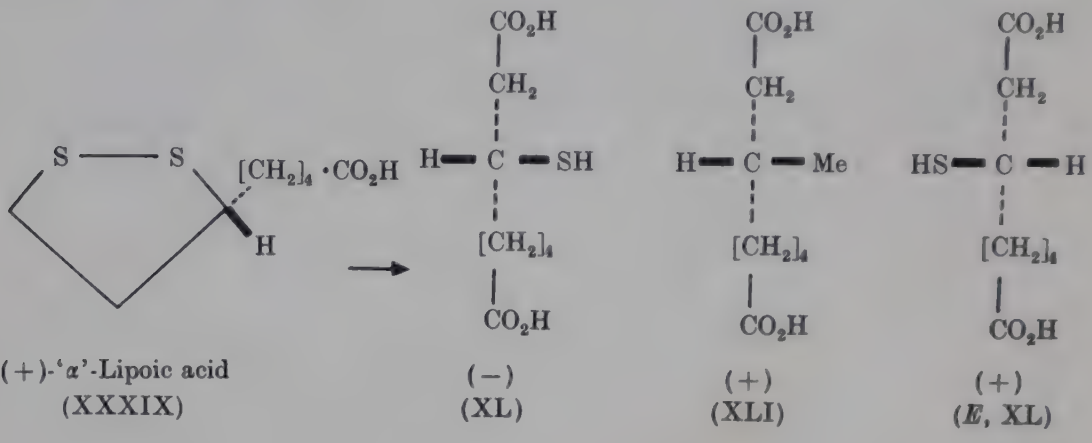


(XXXVIII)
(−)-Mercaptosuccinic acid

lation of (+)-methylsuccinic acid (XXXVII) and (−)-mercapto-succinic acid (XXXVIII), which give a quasi-racemic compound, and are therefore of opposite configuration. This, with other similar correlations, was the first link between branched-chain compounds and the glyceraldehyde series; it was subsequently confirmed by evidence of other kinds (see Mills & Klyne, 1954).

A recent example studied by Mislow & Meluch (1956) applies melting-

point curves in a somewhat different fashion; this concerns 3-mercaptooctanedioic acid (XL) and 3-methyloctanedioic acid (XLI). The compounds (XL) and (XLI) give solid solutions for all mixtures, whilst the enantiomer, (*E*,XL), and (XLI) give a diagram with a single eutectic. This indicates that (XL) and (XLI) are of the same absolute configuration whilst (*E*,XL) and (XLI) are of opposite configurations (cf. Mislow & Heffler, 1952). The thio acid (XL) is related to (+)-'α'-lipoic acid (XXXIX). Other examples are to be found in work on diphenic acids (Newman *et al.* 1958).



(+)-'α'-Lipoic acid (XXXIX)

(−) (XL)

(+) (XLI)

(+) (*E*, XL)

X-RAY-CRYSTALLOGRAPHIC METHODS

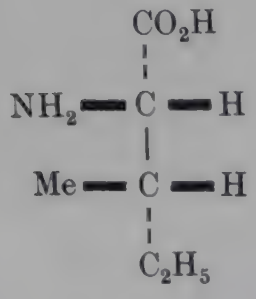
The uses of the X-ray-diffraction technique for correlations are of two kinds: (i) for absolute configurations and (ii) for relative configurations.

X-ray-crystallographic methods do not, in general, distinguish between enantiomers. An ingenious technique due to Bijvoet (Utrecht) employs X-radiation of a wavelength which is partly absorbed by an atom in the crystal under examination, and permits a solution of the phase problem, i.e. determines the absolute configuration of the structure (for reviews see Bijvoet, 1955; Speakman, 1957). This method, first applied by Bijvoet *et al.* (1951) to sodium rubidium tartrate, showed that by a fortunate chance the Fischer convention for glyceraldehyde is correct. Subsequent applications of this technique are to isoleucine (XLII; Trommell & Bijvoet, 1954), strychnine (XLIII; Peerdeman, 1956) and a cobalt complex (Saito, Nakatsu, Shiro & Kuroya, 1955).

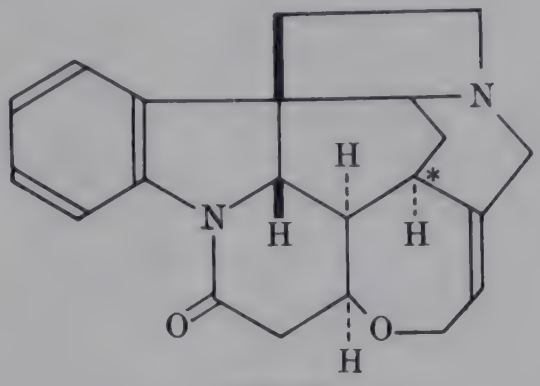
X-ray-crystallographic studies by the 'ordinary' techniques (as distinct from the special method of Bijvoet) are often of great value in giving a decisive correlation between two asymmetric centres. Important earlier studies are those of threonine and hydroxyproline, of cholesteryl iodide (which provides the most reliable evidence for many details of steroid stereochemistry) and of penicillin (which gives some steric detail

which would be difficult, if not impossible, to obtain otherwise). (For references see Mills & Klyne, 1954.)

An outstanding recent example is the work of Hodgkin and her colleagues (Hodgkin *et al.* 1957) on vitamin B_{12} (XLIV); here the known



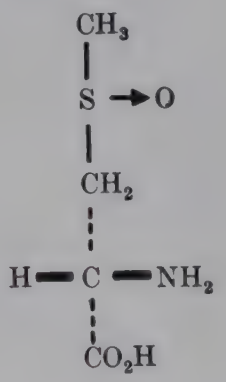
(XLII)
L-Isoleucine



(XLIII)
Strychnine

* This atom corresponds to C-15 in the yohimbé alkaloids (cf. XII and XIV).

absolute configuration of ribose may be considered as a reference point from which are obtained the absolute configurations of the 1-aminopropan-2-ol unit, the asymmetric cobalt atom, and the modified and reduced porphyrin system. Another simpler but valuable correlation is that of Hine & Rogers (1956), who studied *S*-methyl-L-cysteine sulphoxide (XLV); they obtained the absolute configuration of the asymmetric sulphur atom by reference to the known configuration of the α-amino group in cysteine.



(XLV)
S-Methyl-L-cysteine sulphoxide

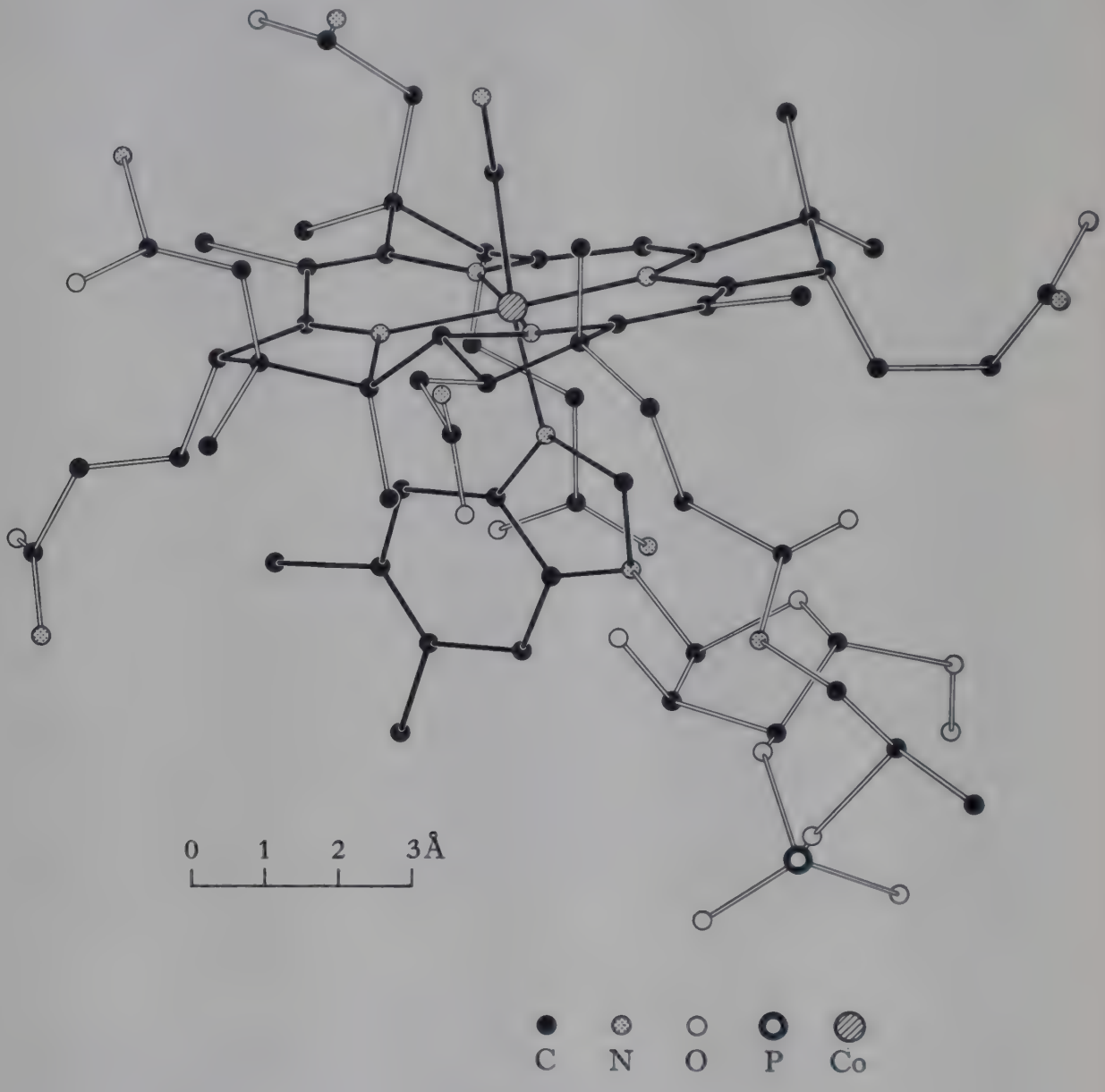
OPTICAL ROTATIONS

Any physical property is useful in structural studies only if it can be divided in some way into units associated with different atoms or groups. In work on optical rotations we choose individual centres of asymmetry or groups of centres, and we try to allot values to them. All arguments are based on *analogy*, and the usefulness of arguments depends on the care with which analogies have been chosen. Wherever rotational arguments have subsequently been proved wrong, it is because of bad analogies. (For a detailed review see Klyne, 1955.)

Until recently, nearly all work on optical rotations has been carried out at a single wavelength. Recent instrumental developments have permitted the measurement of complete rotatory-dispersion curves (i.e. rotation–wavelength curves), which in the future will probably take the place of monochromatic rotations. In this review arguments

based on monochromatic rotations are considered first, and the newer dispersion work afterwards.

Comparison must always be made between values for the same wavelength and in the same (or similar) solvent. All values in this review are



(XLIV)

Vitamin B_{12}

This diagram is reproduced by kind permission of Dr D. M. C. Hodgkin, F.R.S., and of the Council of the Royal Society.

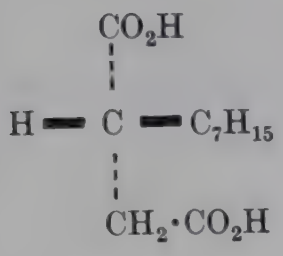
molecular rotations, and are for the sodium D line (589 mμ), unless otherwise stated.

Mol. rotation $[M]$ or $[\phi]$ = specific rotation $[\alpha] \times$ mol. wt./100.

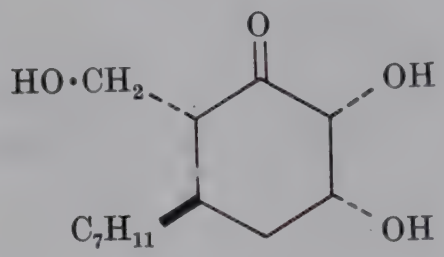
Monochromatic rotations. One general principle is at the basis of all arguments about optical rotations, namely the principle of the inde-

pendence of asymmetric centres. Shortly after Van't Hoff put forward the idea of the tetrahedral carbon atom he suggested that, in a compound which has two or more asymmetric centres, the rotation contributions of the individual centres are additive. This principle is rarely correct in quantitative form, because of the mutual effects of asymmetric centres (*vicinal action*). Much of the interest of optical rotation work lies in the study of such vicinal action.

Two qualitative expressions of the idea of independence of asymmetric centres are the following: Freudenberg's Rule of Shift (Verschiebungssatz) states that if two related structures of the same configuration A and B are altered in the same way to give A′ and B′, then the molecular-rotation differences (A′ − A) and (B′ − B) will be of the same sign. The Distance Rule (Entfernungssatz) of Tschugaeff states that, in an homologous series of compounds of the same configuration, $[M]$ tends to a limiting value as one ascends the series.



(XLVI)



(XLVII)
Palitantin

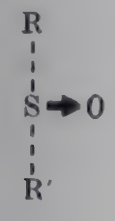
Some recent examples of the application of rotations in homologous series are the following:

(+)-*n*-Heptylsuccinic acid (XLVI) is very probably the D-isomer, as shown, since all other (+)-*n*-alkylsuccinic acids have the same configuration (Fredga, 1942). This permits the allotment of configuration to palitantin (XLVII; Bowden, Lythgoe & Marsden, 1959; Birkinshaw & Raistrick, 1936; Birkinshaw, 1952).

The X-ray work of Hine & Rogers (1956) has shown that the absolute configuration of the sulphoxide group in *S*-methyl-L-cysteine sulphoxide is as shown in (XLV). This compound has a large positive rotation, $(M_D + 177)$ which can be ascribed chiefly to the sulphoxide group, since *S*-methylcysteine itself has $M_D - 43$. It seems reasonable (cf. Hine & Rogers, 1956; Kjaer, 1959) to suggest that the numerous naturally occurring sulphoxides which have either large positive or large negative rotations have the respective configurations (XLVIII) and (XLIX); however, since there is a remote possibility that vicinal action, by the α-amino acid group, influences the rotational contribution of the sulph-

oxide in (XLV), these allotments of configuration should not be considered as completely definite.

Rotational arguments are particularly convincing when the differences in rotation between two possible configurations are large. Good examples are provided by many types of glycosides. The difference in M_D values between α-D- and β-D-glycosides of general types (L) and (LI) is usually of the order of +400, and configurations at glycosidic linkages can often be allotted with certainty by using rotational evidence.



(+)

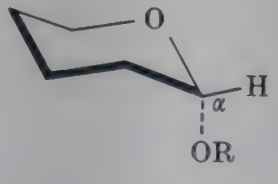
(XLVIII)

(−)

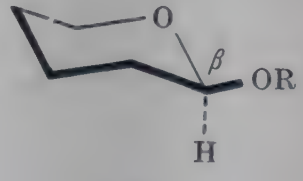
(XLIX)

(R < R')

The three possible isomers of trehalose (the non-reducing disaccharides formed of two glucopyranoside units) have widely differing rotations: $\alpha\alpha'$, +674; $\alpha\beta'$, +315; $\beta\beta'$, −156. Recently Arcamone & Bizioli (1957) obtained from a streptomycete a non-reducing disaccharide consisting of one glucose and one glucosamine unit (M_D +665). The rotations of methyl glucosides and glucosaminides are not greatly different. The rotation of the new disaccharide can therefore be taken as good evidence that its glycosidic link is $\alpha\alpha'$.



(L)



(LI)

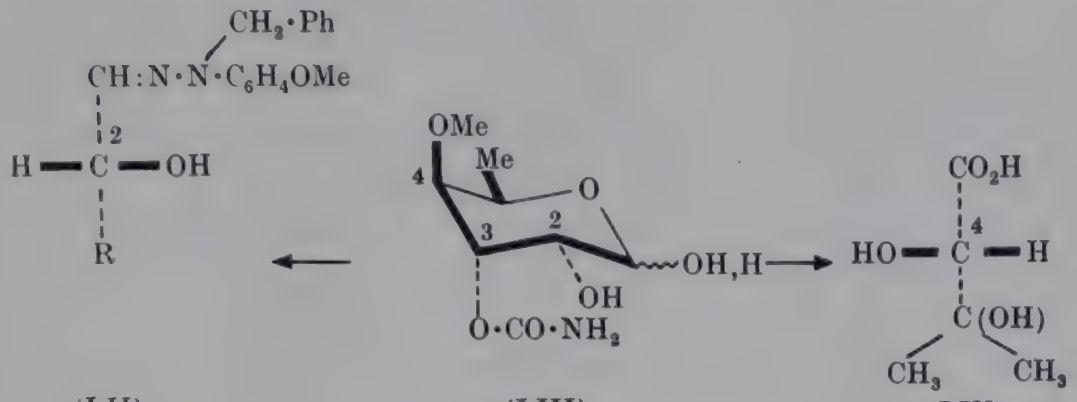
For glycerides of optically active aglycones (Z) it is approximately true that

M_D(Zα- or Zβ-glycoside) = M_D(Z) + M_D(methyl α- or β-glycoside).

This empirical rule (Klyne, 1950) has been much used to allot the anomeric configurations of the sugars in the cardiac glycosides, and in glycoalkaloids (Staněk, 1956).

Rotational evidence may be very helpful, even when it does not prove a configuration conclusively, by indicating the most probable structure and therefore the most profitable lines for further chemical work. A good example is provided by the new sugar 3-*O*-carbamylnoviose,

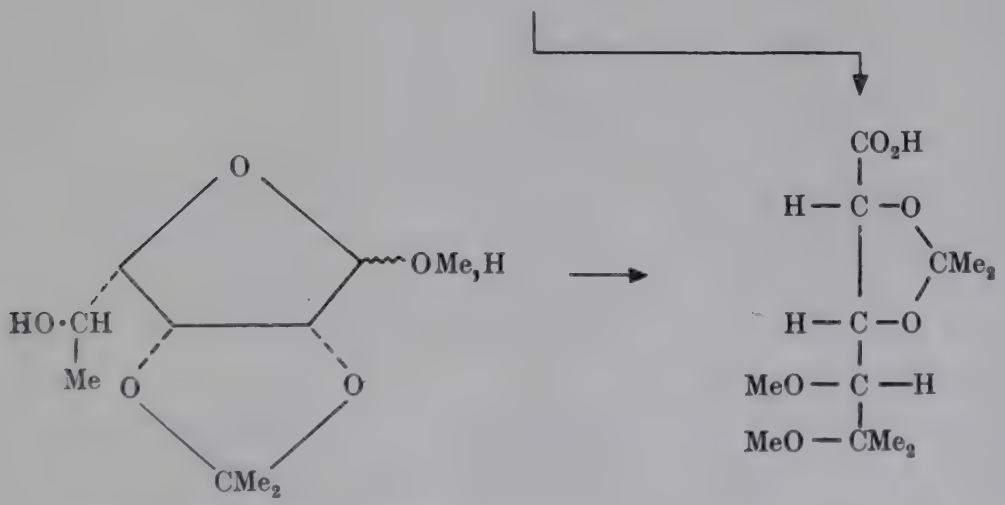
one of the units of the antibiotic novobiocin. The Merck group (Walton, Rodin, Stammer, Holly & Folkers, 1956) suggested that the stereochemistry of this compound is as shown in formula (LIII). Two separate rotational arguments were used to allot configurations at C-2 and C-4.



(LII) (LIII) (LIV)

The hydroxyl group at C-2 must be D because the *N*-benzyl-*N*-*p*-methoxyphenylhydrazone (as LII) is laevorotatory (hydrazone rule, Votoček, 1931). The hydroxyl group at C-4 must be L because the acid (LIV, derived from LIII) is dextrorotatory, whilst its alkali salts are laevorotatory (Clough, 1915, 1918; Levene & Meyer, 1916). The configuration at C-3 was allotted on chemical grounds.

3-*O*-Carbamylnoviose



Methyl 2:3-*iso*propylidene-L-rhamnofuranoside (LV)

(LVI)

For a compound of some potential importance it was clearly desirable that these arguments should be checked by strict chemical evidence and this has now been done. Recently another paper from the same Laboratory (Walton, Rodin, Stammer, Holly & Folkers, 1958) has described the synthesis from the L-rhamnose derivative (LV) of an acid (2:3-*iso*propylidene-5-*O*-methylnovionic acid, LVI) also obtained from novobiose.

Errors: use of false analogies. The most famous example of an error due to the misuse of optical-rotation evidence is Hudson's allotment of the furanose configuration to many common sugars which were shown by the degradations of the Birmingham school (Haworth & Hirst) to be of pyranose type. An excellent historical account of this error—and of Hudson's very generous admission of it—is given in the Hudson Memorial Lecture by Hirst (1954).

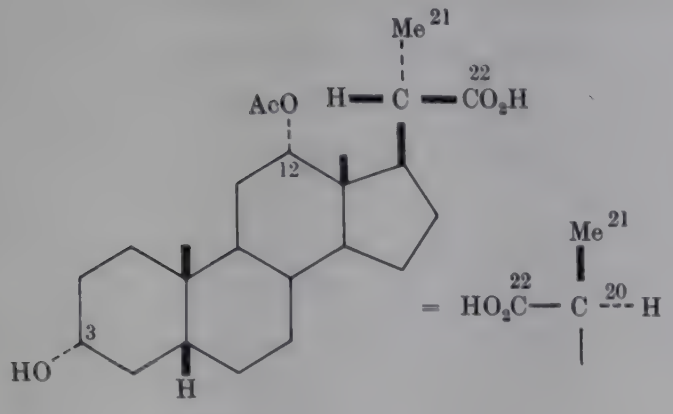
On several occasions errors have arisen from false analogies between cyclic and acyclic compounds. Examples are as follows: (i) The allotment of the wrong absolute configuration to the steroids by Stokes & Bergmann (1952), who wrongly compared rotational differences of cyclic and acyclic alcohols. This work was subsequently corrected by Mills (1953). (ii) The allotment of the wrong absolute configuration to morphine by Bick (1952), who argued from analogies between aporphine and 'open' benzyltetrahydro*iso*quinoline derivatives. This was corrected by Bentley & Cardwell (1955) and also by degradational work (Kalvoda, Buchschacher & Jeger, 1955).

An instance where the right answer has been reached, though on completely incorrect reasoning, is provided by a recent paper on the steroid alkaloid conessine by Phillips & Johnson (1957). These authors noted that dihydroconessine ($M_D + 165$) was much more dextrorotatory than its hetero-isomer (epimeric at C-20; $M_D + 54$). By analogy with two derivatives of bisnordeoxycholic acid [20-*n*, as (LVII), $M_D + 144$; and 20-*iso*, as (LVIII), $M_D + 38$] they suggested that the two alkaloidal derivatives were as (LIX) and (LX) respectively.

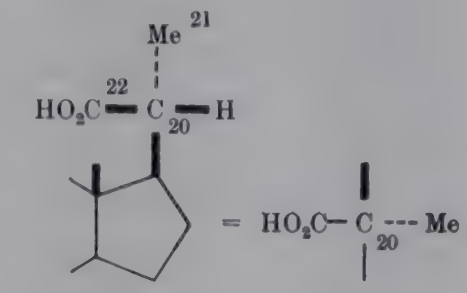
In fact, the configurations (LIX) and (LX) have been proved by chemical methods (Černý, Lábler & Šorm, 1957). The rotational analogies are false because (*a*) a tertiary nitrogen attached to C-20 in the alkaloids is in no way analogous to the carboxyl group in the acids, (*b*) C-20 is part of a ring in the alkaloids, but not in the acids (cf. Haworth & McKenna, 1957).

Analogies between five-membered and six-membered rings may also be dangerous. The allotment of configurations to the necine bases by Leonard (1957), which was based on such an analogy, was proved wrong by the work of Warren & Klemperer (1958) and of Adams & Fleš (1959*a*, *b*).

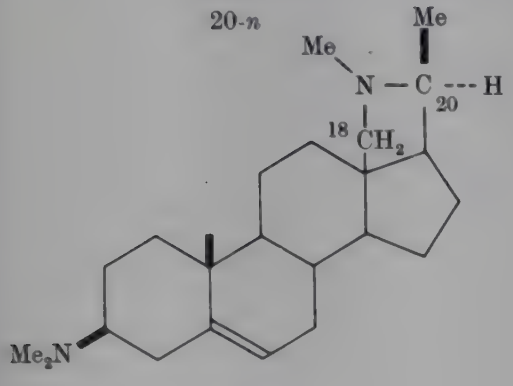
Rotatory dispersion. A rotatory-dispersion curve is one in which the optical rotation of a compound is plotted against the wavelength of light. The application of rotatory-dispersion measurements as a tool in structural and stereochemical studies is recent; the sudden growth of this work is due to the commercial introduction of a spectropolarimeter (Rudolph, 1955) which permits the measurement of a curve from 700 to about 270 mμ in about 1 hr. With one of these instruments, Djerassi



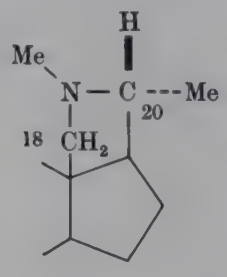
(LVII)
20-*n*



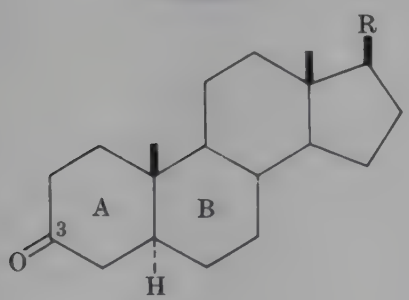
(LVIII)
20-*iso*



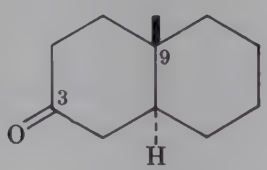
(LIX)
Conessine



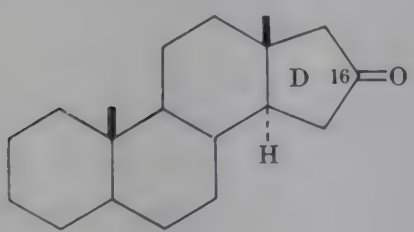
(LX)
Heteroconessine



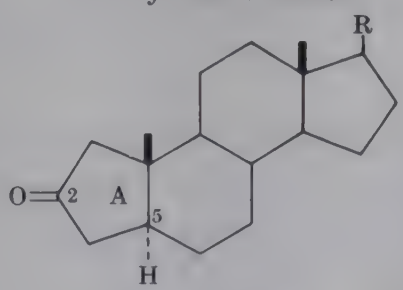
(LXI)
3-Oxo 5α steroid



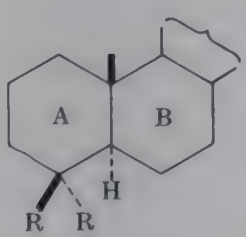
(LXII)
9-Methyl-*trans*-3-decalone



(LXIII)
16-Oxo steroid



(LXIV)
2-Oxo 5α A-nor steroid



(LXV)

(Wayne State University, Detroit, and Stanford University) and his colleagues have in five years measured about 2000 curves (for reviews see Djerassi, 1960; Klyne, 1960*a*, *b*). Within the last two years a number of other Laboratories, including the author's, have become active in this field.

Classical work (for summaries see Lowry, 1935; Levene & Rothen, 1938) showed that compounds which do *not* absorb light within the observed spectral range give smooth dispersion curves; the rotation $[M]$ (or $[\phi]$ to use the newer symbol) usually increases in magnitude towards lower wavelengths, and the curves usually have no maximum

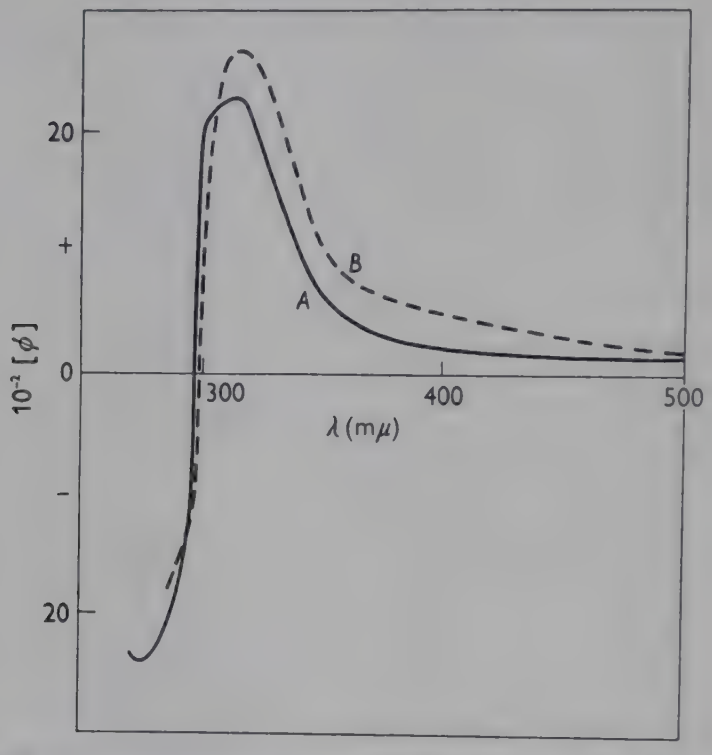


Fig. 3. Rotatory-dispersion curves. *A*, 5α-cholestan-3-one (LXI); *B*, 9-methyl-*trans*-3-decalone (LXII).

or minimum. Compounds which *do* absorb light within the observed spectral range give more complex curves, usually with one or more pronounced maxima and minima. ('Cotton effect curves'; for nomenclature see Djerassi & Klyne, 1957.) The carbonyl group, as ketone or aldehyde ($\lambda_{max.}$ at 280–300 mμ; $\epsilon \sim 50$), has proved the most convenient absorbing group for this purpose.

Djerassi and his colleagues first examined a complete range of steroid ketones with ketone groups in all possible positions and found that each position has its characteristic type of curve. Many bicyclic ketones—analogous to common steroid types, and of known absolute configuration—were then examined, and with few exceptions each gave a Cotton effect curve of the *same* sign as its steroid analogues (see Fig. 3 and formulae LXI, LXII); this provided a sound basis for the generalization

(Djerassi *et al.* 1956) that 'typical features of dispersion curves of alicyclic mono-ketones are on the whole only a reflexion of the immediate structural and stereochemical environment around that particular carbonyl group'. The generalized method of molecular-rotation differences (Klyne, 1952, 1953*b*) can therefore be extended to rotatory-

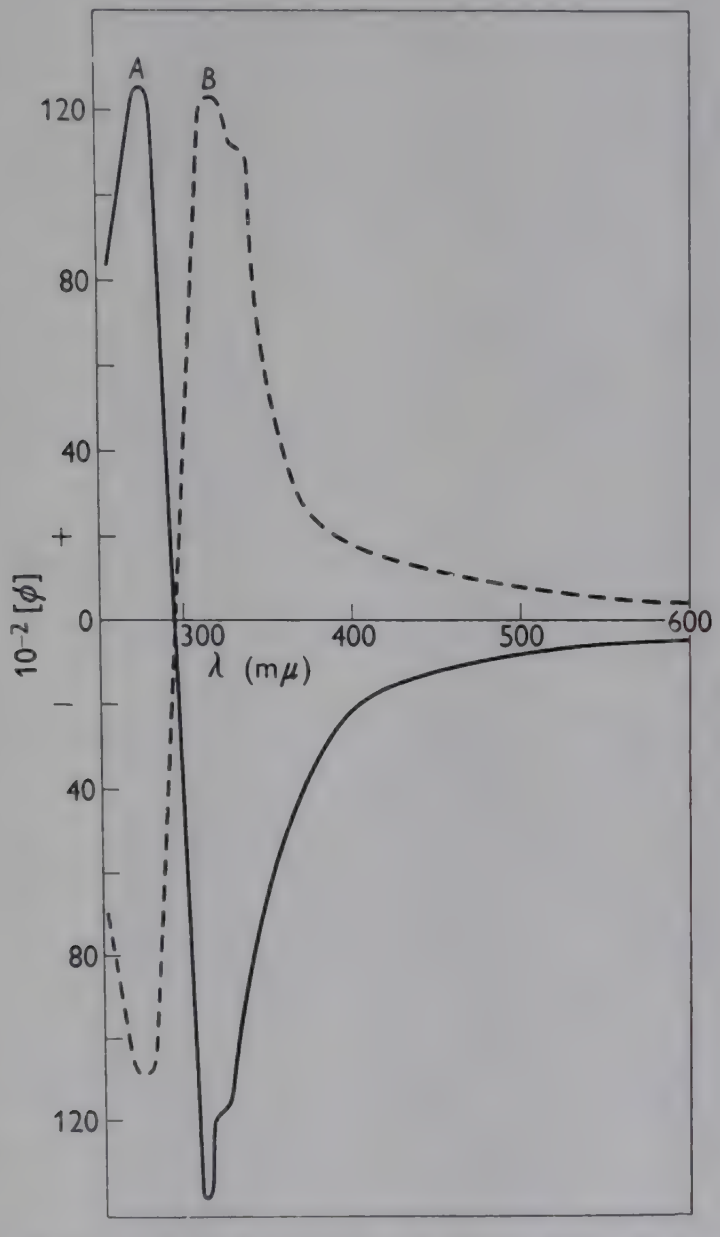
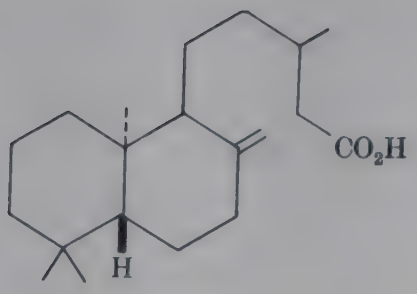


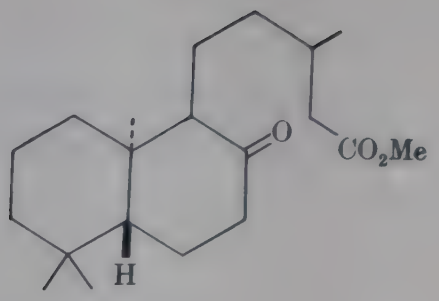
Fig. 4. Rotatory-dispersion curves. *A*, 16-oxo steroid (LXIII); *B*, 2-oxo 5α A-nor steroid (LXIV).

dispersion curves as follows (Djerassi *et al.* 1956): '(*a*) Terminal ring units of the same type make contributions to the rotatory dispersion curve which are, very approximately, independent of the nature of the rest of the molecule. (*b*) Each terminal unit can exist in two enantiomeric types which have rotatory dispersion contributions of opposite sign.'

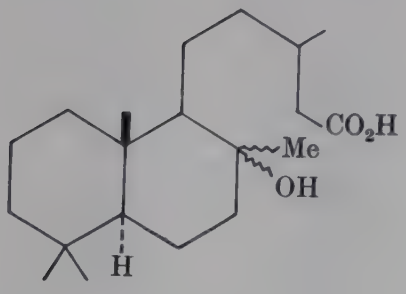
An excellent illustration of the second principle is provided by 16-oxo and A-nor 2-oxo steroids (LXIII, LXIV), the dispersion curves of which (Fig. 4) are almost exactly mirror-images.



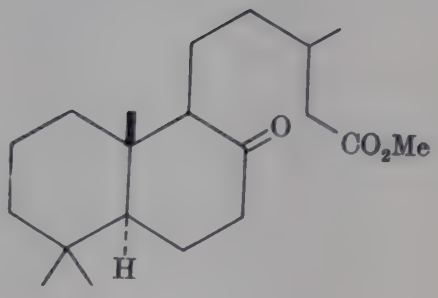
(LXVI)
Eperuic acid



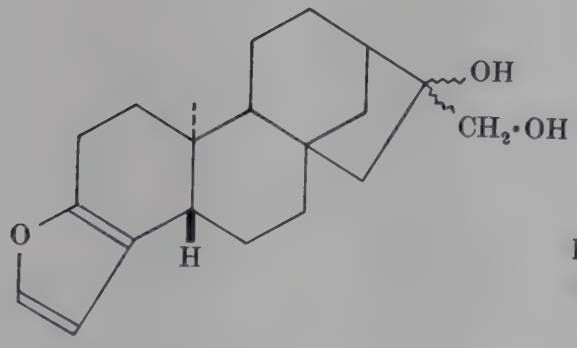
(LXVII)



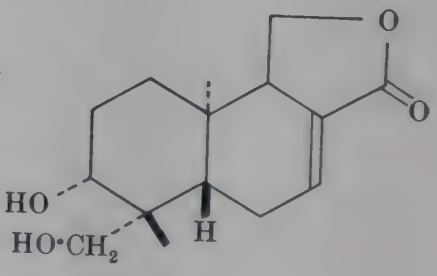
(LXVIII)
Labdanolic acid



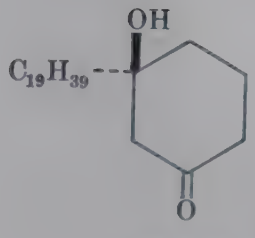
(LXIX)



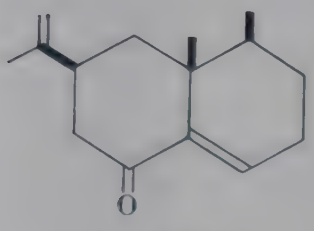
(LXX)
Cafestol



(LXXI)
Iresin



(LXXII)



(LXXIII)
Eremophilone

All steroids, triterpenes and diterpenes whose structure was known up to 1955 had the same absolute configuration at the key point C-10 (the

angle methyl group between rings A and B)* (LXV; R, R generally = H, H or Me, Me).

Table 1. *Allotment of configurations by rotatory-dispersion evidence*

Compound to which configuration is allotted	Reference compounds of known configuration	References
Cafestol (LXX)	4α-Ethyl-5α-cholestan-3-one	Djerassi, Cais & Mitscher (1958, 1959)
Iresin (LXXI)	4α-Methyl-5α-cholestan-3-one	Djerassi & Burstein (1958)
Alantolactone derivatives	9-Methyl-*trans*-2-decalone and cholest-4-en-3-one	Djerassi (1958, unpublished work); Benesova, Sýkora, Herout & Šorm (1958)
ψ-Santonin	9-Methyl-1-decalones	Djerassi, Riniker & Riniker (1956); Sumi, Dauben & Hayes (1958); Cocker, McMurry & Hopkins (1959)
Cadinol derivatives	Decalones	Herout & Sýkora (1958)
trans-1-Decalone	Octant Rule, also asymmetric synthesis	Feldman & Prelog (1958); Baumann & Prelog (1958*a*, *b*)
Hydroxydecalone derivatives	9-Methyldecalones	
α-Cyperone and carissone	Cholest-4-en-3-one	Djerassi *et al.* (1956)
4-Methyl-6-oxoheptanoic acid	4-Methylheptan-2-one	Eisenbraun, Osiecki & Djerassi (1958)

For other examples see Djerassi (1960).

The diterpene acid, eperuic acid (King & Jones, 1955), was the first higher terpene to be found which, whilst following the general structural pattern of diterpenes, triterpenes and steroids, has the 'abnormal' stereochemistry at C-10 (steroid numbering) shown in (LXVI). The dispersion curves (Djerassi & Marshall, 1957) of very similar ketones (LXVII, LXIX) obtained from eperuic acid on the one hand and from labdanolic acid (LXVIII) (Cocker & Halsall, 1956) on the other hand show clearly that eperuic acid has the 'abnormal' configuration at C-10; (LXVII) shows a positive Cotton-effect curve, whilst (LXIX), the absolute stereochemistry of which is known, shows a negative curve, which is almost, but not exactly, enantiomeric with that of (LXVII).

Other examples where absolute configurations have been allotted or confirmed by analogies using rotatory-dispersion evidence are summarized in Table 1.

The following points are worthy of comment. Cafestol (LXX) and iresin (LXXI) (in Table 1), also farnesiferol A (Caglioti, Naef, Arigoni & Jeger, 1958) and probably maaliol (Büchi, Schach v. Wittenau & White, 1959) are further examples of terpenes with 'abnormal' stereochemistry at C-10 (cf. eperuic acid).

The allotment of configuration of 3-hydroxy-3-nonadecyl*cyclo*-

* Steroids having an aromatic A or B ring, e.g. the oestrogens, have no asymmetry at C-10; however, their configurations at the other angle-methyl position (C-13) agree with all other naturally occurring steroids.

hexanone (LXXII) (Lamberton, 1958) is made with the use of a new semi-theoretical generalization regarding the Cotton effects of ketones, the Octant Rule (Moffitt, Moscowitz, Woodward, Djerassi & Klyne, 1959) (cf. Djerassi, 1960; Klyne, 1960*a*, *b*).

The stereochemistry of the 'irregular' sesquiterpene eremophilone has been the subject of much discussion. The latest results (Zalkow, Markley & Djerassi, 1959) show conclusively that the absolute configuration is as shown in formula (LXXIII).

Whilst plain dispersion curves offer less striking evidence of configurational relations than do Cotton-effect curves, they are at least much better than the old-fashioned 'monochromatic' measurements. Examples are provided by recent work on emetine (van Tamelen & Hester, 1959) and in the flavans (Birch *et al.* 1957). Systematic studies of plain curves in the steroid field are in progress in the author's Laboratory (Jones & Klyne, 1960); cf. also the important work of Sjöberg (1960) on carboxylic acids.

ENZYMIC EVIDENCE

Evidence from stereospecific enzymic reactions can often be of value for relating configurations to a known standard. Since several later papers in this Symposium deal with stereospecificity of enzymes, this method will not be considered here.

It is sufficient to point out that the choice of analogies is again very important; perhaps the most reliable enzyme correlations are those within a homologous series (see, for example, Greenstein, 1954, on amino acids) or within closely related groups of glycosides.

CONCLUSION

It is hoped that this review has shown what important and fascinating studies are to be found in the field of correlations. Although much has been done in the past five years, a wide field still remains to be covered.

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DISCUSSION OF PAPER BY KLYNE

Olga Kennard (*National Institute for Medical Research, Mill Hill*): An important contribution to the correlation between structure and activity of biological products was recently published by Dr H. Sörum (*Acta chem. scand.* **13**, 345, 1959), who determined the molecular and

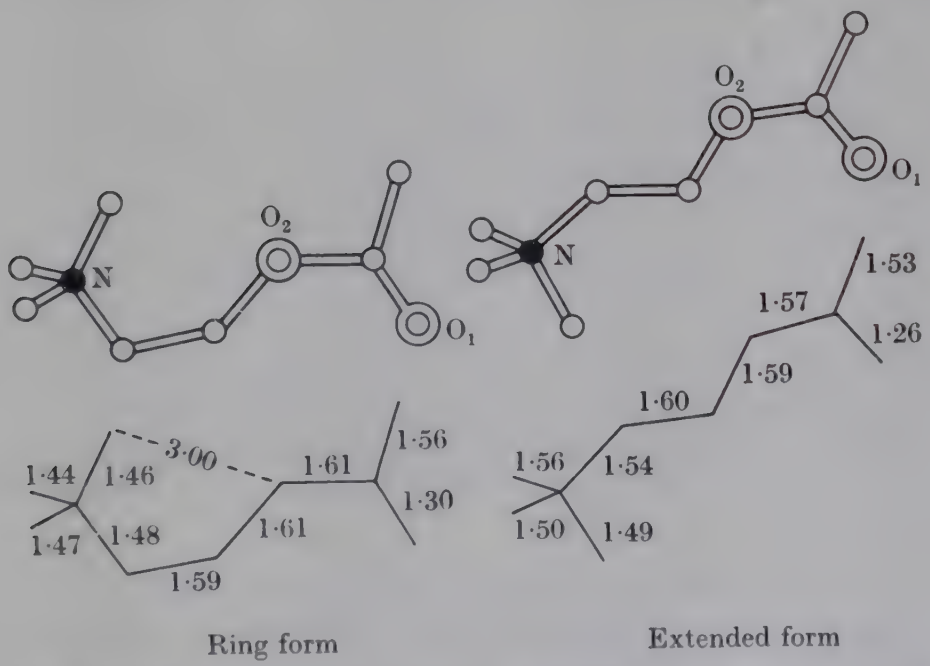

Fig. 1. Shape and dimensions of the two acetylcholine ions in acetylcholine bromide (bond distances in Å).

crystal structure of the acetylcholine ion. He finds that the molecule exists in two distinct configurations in the same crystal structure: an extended form as previously assumed on chemical evidence, and a 'ring' form in which the methyl group of the choline radical approaches to within less than 3 Å the ester-oxygen atom (Fig. 1). A similar 'ring' structure was reported in muscarine iodide by Jellinek (*Acta cryst., Camb.*, **10**, 277, 1957) and is shown in Fig. 2 for comparison.

The existence of two significantly different molecular configurations in the same crystal is most unusual and indeed has not previously been reported [cf. Interatomic Distances, *Chem. Soc. spec. Publ.*, no. 11, 1958: *M* 131 (Dithio-oxamide); *M* 233 (*trans*-Azobenzene); *M* 251 (Dibenzophenathrene); *M* 257 (Bisdiphenylene-ethylene)].

An examination of the arrangement of the acetylcholine ions in the crystal structure shows no evidence that the difference in the molecular forms is produced by packing, or van der Waals forces, and it seems reasonable to assume that the two configurations also exist in solutions.

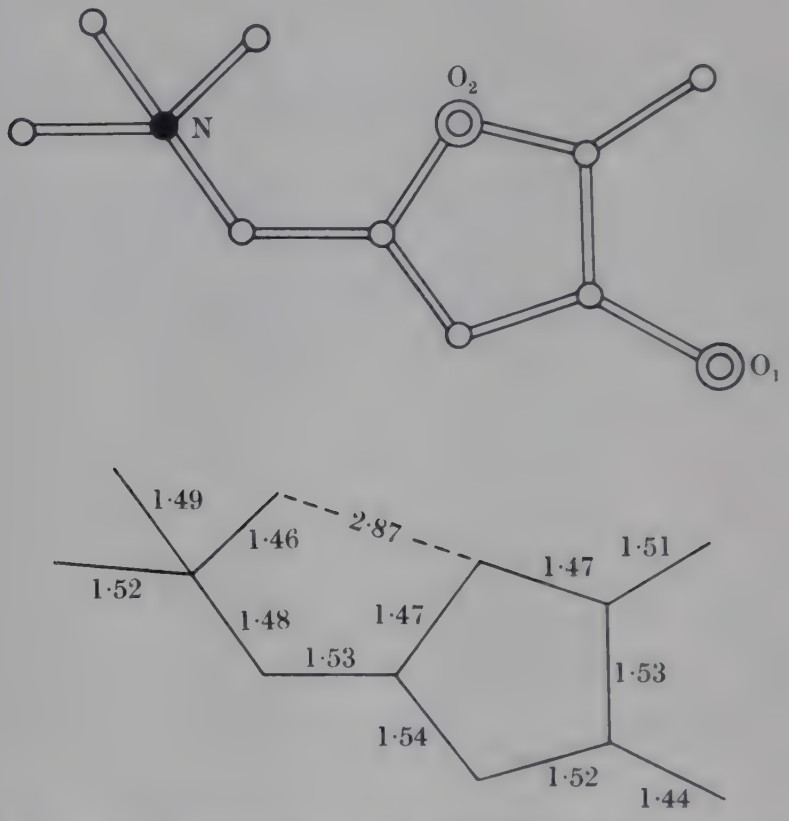

Fig. 2. Shape and dimensions of the muscarine ion in muscarine iodide (bond distances in Å).

The extended form may be predominant in polar, and the 'ring' form in non-polar media, and may explain the behaviour of acetyl choline at lipid–water interfaces. Further, the existence of the unusual 'ring' structures in both acetylcholine and muscarine suggest that the molecular configuration may have an important bearing on the physiological action of these substances.

Dr A. H. Beckett (*Chelsea College of Science and Technology*): A new method of configurational assignment was briefly reported by Beckett & Anderson (1957). This method may be of special value when unequivocal chemical synthetic routes are precluded. It involves the prepara-

tion of stereoselective adsorbents: silica gel is allowed to form slowly in the presence of molecules of a suitable reference substance, the gel is allowed to age and is then dried, powdered and extracted to remove the enmeshed organic reference molecules from the surface layers of the gel to leave molecular imprints of the desired configuration. These stereoselective adsorbents adsorb molecules of similar configuration more readily than those of dissimilar configuration, provided that their structures are not too different from those of the molecules used to make the molecular imprint in the gel; e.g. a quinine-selective adsorbent adsorbs cinchonidine (like configuration) more readily than its stereoisomer cinchonine, whereas a quinidine-selective adsorbent adsorbs cinchonine better than cinchonidine.

This method has been shown to be applicable to diverse molecules, e.g. tropine alkaloids, morphine-type structures, isomeric piperidines.

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Dr W. B. Whalley (*Chemistry Department, The University, Liverpool*): The configuration of *epi*catechin (unlike that of catechin) is still under discussion. The work of Birch, Clark-Lewis & Robertson (1957) indicated that (−)-*epi*catechin had the $2R{:}3R$ configuration. This result does not appear to be well founded since the application of the Prelog atrolactic method to catechin and *epi*catechin is open to grave criticism. Furthermore, the recent work of Brown & Somerfield (1958), in which results completely opposed to those of Birch *et al.* have been obtained, indicates that the problem of the absolute configuration of the *epi*catechins is still unresolved.

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STERIC ASPECTS OF THE BIOSYNTHESIS OF TERPENES AND STEROIDS

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The first experimental evidence pertaining to the biological synthesis of compounds related to the *iso*pentane group of natural products is to be found in the observation that mice fed with heavy water can incorporate large amounts of the isotope into liver cholesterol and the latter therefore must be built up by union of small units (Rittenberg & Schönheimer, 1937; cf. Sonderhoff & Thomas, 1937). In the meantime much has been learned about the stages which lie between the smallest unit, acetic acid, and cholesterol. At the same time the results of structural

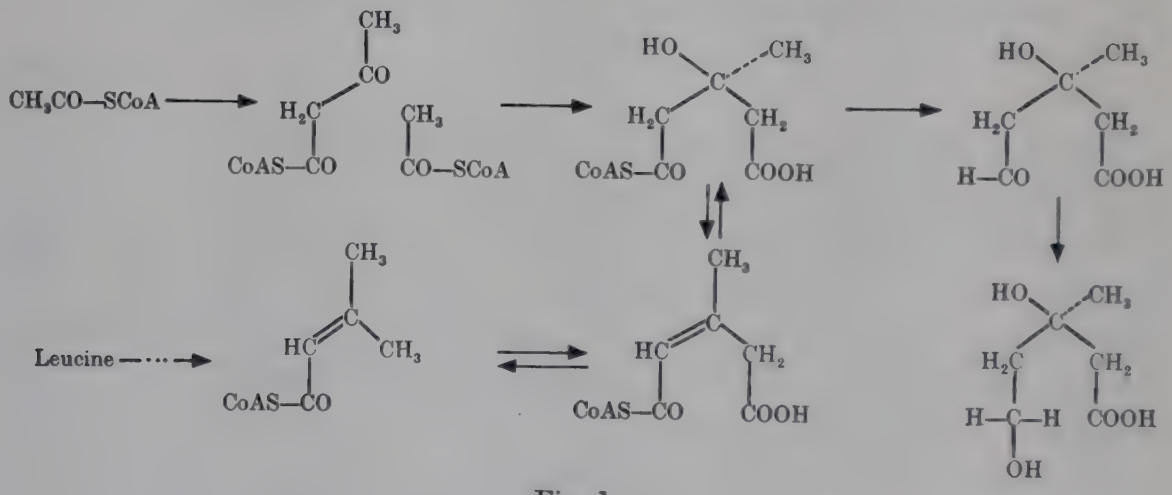


Fig. 1.

investigation of many terpenes of plant origin have led to the hypothesis (Ruzicka, Eschenmoser & Heusser, 1953) that cholesterol biogenesis is only a special case of terpene biogenesis. The suggestion was made that the complicated polycyclic terpene compounds are derived biologically from aliphatic precursors by a process of cyclization, occasionally connected with rearrangements of hydrogen and carbon atoms. The whole biosynthetic process can thus be subdivided into three definite steps: (*a*) formation of a biological *iso*pentane unit from acetate; (*b*) condensation of the biological *iso*pentane unit with formation of aliphatic terpenes; (*c*) transformation of the aliphatic precursors into cyclic derivatives. The present paper is concerned with the discussion of specific steric problems connected with each of these steps.

The early stages of terpene biosynthesis are summarized in Fig. 1.

Two moles of acetyl-CoA first condense to give acetoacetyl-CoA and this reacts with a third mole to form the monoester of hydroxymethylglutaric acid (HMG-CoA) (Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958*b*; Rudney, 1959). A TPNH-dependent reduction of HMG-CoA affords the well-known mevalonic acid (MVA), possibly through the intermediate formation of mevaldic acid or of an enzyme-bound form thereof (Ferguson, Durr & Rudney, 1958; Lynen, 1959). The last three compounds contain an asymmetrically substituted carbon atom and it appears likely that, as in most enzyme-catalysed reactions, their formation is specific in so far as only one of the two possible enantiomers is formed. This hypothesis is supported by the following observations: (*a*) The original isolation of MVA from natural sources (Wolf *et al.* 1957)

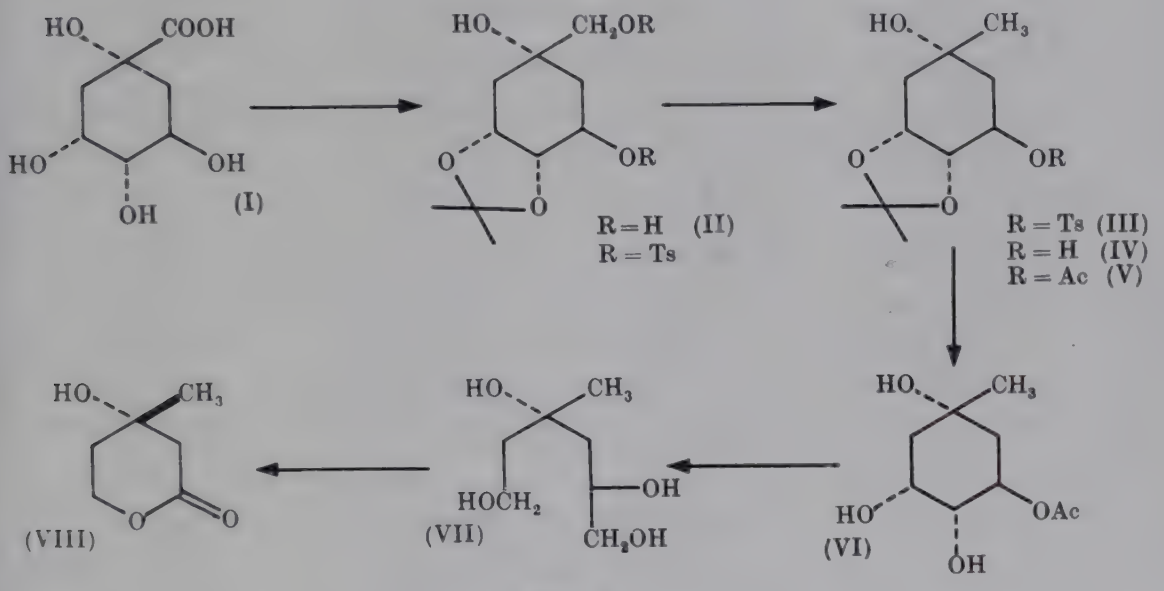


Fig. 2.

yielded a compound with a well-defined optical activity. (*b*) Synthetic racemic samples of MVA exhibit only half of the biological activity of the natural isomer (Wolf *et al.* 1957). (*c*) After feeding of racemic MVA to rats or to Mycobacteria nearly half of the material can be recovered as the biologically inactive enantiomer of MVA (Gould & Popják, 1957; Lynen & Grassl, 1958).

The absolute configuration of the natural enantiomer of MVA has been recently established (M. Eberle & D. Arigoni, 1959, unpublished work) through a direct correlation with quinic acid, previously related to the system of glyceraldehyde (Dangschat & Fischer, 1950), as illustrated in Fig. 2. Quinic acid (I) was first converted into the acetal and then reduced with lithium aluminium hydride to the triol (II). Tosylation of (II), followed by reduction with lithium aluminium hydride, afforded the monotosylate (III), readily converted into the alcohol (IV) by the use of sodium amalgam. Acid hydrolysis of the

corresponding acetate (V) liberated a glycol group (cf. VI), which was then cleaved with periodic acid. Reduction of the resulting dialdehyde with lithium aluminium hydride produced a new glycol (VII), which was again cleaved by periodic acid. Oxidation of the cleavage product with bromine gave an oily product which was characterized through a crystalline derivative and proved to be identical with the biologically inactive enantiomer (VIII) of the lactone of MVA. The natural isomer and the related acids must therefore possess the configuration shown in Fig. 1. Apart from its intrinsic value, knowledge of the absolute configuration of MVA might prove useful in a different context, as is illustrated below.

Before turning to the further biological transformation of MVA it may be recalled that there is an alternative route for the formation of HMG-CoA which starts with the amino acid leucine and leads through the CoA esters of β-methylcrotonic acid and β-methylglutaconic acid (Fig. 1) (Hilz, Knappe, Ringelmann & Lynen, 1958; Lynen, 1959; Coon, Kupiecki, Dekker, Schlesinger & del Campillo, 1959). While it is still not known whether the *cis* or the *trans* form of the glutaconic ester is the natural substrate of this sequence, the hydration of the double bond must be stereospecific as far as the addition of the hydroxyl group is concerned. At the present time it is not known if this applies to the addition of the proton as well. In the absence of a *cis–trans* isomerase for the β-methylglutaconic acid it is very likely that even the carboxylation in which the acid is produced takes place specifically at one of the two methyl groups. One further point of the sequence in which stereospecificity is expected is the TPNH-dependent reduction of mevaldic acid. By analogy with the experiments of Westheimer & Vennesland (cf. Vennesland, 1958) on different dehydrogenases one is inclined to think that such reaction would result in the production of a well-defined configuration of the two hydrogen atoms at C-5 of the MVA molecule. Though the formation of mevaldic acid has admittedly not yet been demonstrated in biological systems, one of its enantiomers is known to be reduced by a specific enzyme (Lynen, 1959), and this reaction might therefore be used for the production of MVA specifically labelled at C-5.

We next have to consider the reactions which lead from MVA to the aliphatic terpenes (Fig. 3). After phosphorylation of the primary hydroxyl group (Tchen, 1958; Bloch, 1959; Chaykin, Law, Phillips, Tchen & Bloch, 1958; Lynen, 1959) the carboxyl and the tertiary hydroxyl group are eliminated in a simultaneous process (Rilling, Tchen & Bloch, 1958) with the formation of *iso*pentenyl pyrophosphate (VIII), the long-sought biological *iso*pentane unit. According to Agranoff, Eggerer, Henning & Lynen (1959) a special isomerase can

transform *iso*pentenyl pyrophosphate to $\alpha\alpha$-dimethylallyl pyrophosphate (IX). In this process, the detailed mechanism of which is still obscure, a new methyl group is produced, which has its origin in C-2 of MVA (marked * in Fig. 3). It will be shown in the sequel that enzymes can differentiate between the 'old' and the 'new' methyl group of (IX). This difference must of course be a steric one, and it can therefore be inferred that the isomerization of (VIII) proceeds in a stereospecific manner to produce only one of the geometrical isomers of (IX). To put it in other words, there must be a well-defined geometrical relationship between the methyl group derived from C-2 of MVA and the hydrogen atom at the double bond of (IX). This specificity makes it very probable that only one of the two hydrogen atoms in the methylene group of *iso*pentenyl pyrophosphate is involved in the isomerization.

As a consequence of the allylic position of its ester group, compound (IX) can serve as a starter for the condensation reaction (Rilling *et al.* 1958; Bloch, 1959; Lynen, Eggerer, Henning & Kessel, 1958*a*). Fission of this group gives a cation which can alkylate a unit of *iso*pentenyl pyrophosphate to give a new allylic phosphate ester with 10 carbon atoms, identical with geranyl pyrophosphate (X). Repetition of the same process with the latter leads to the formation of the higher isoprene analogues, e.g. the pyrophosphates of farnesol, geranylgeraniol etc. Again the process is seen to be a stereospecific one, inasmuch as the double bond produced in the reaction usually possesses a *trans* orientation.

Although the available experimental evidence is in accord with the requirements of the scheme (cf., however, the formation of higher branched-chain compounds in which the original carboxyl group of MVA is retained; Popják, 1959; Ogilvie & Langdon, 1959) it must be stressed that the detailed mechanism of the condensation is still a matter of considerable speculation. By analogy with the process of biological methylation it has been pointed out (Birch & Smith, 1959) that, after the fission of the allylic group in (IX), the cation could be first transferred to a sulphur-containing, possibly enzyme-bound substance (XI), which actually represents the true alkylating reagent in the condensation. This is supported by the observation (Popják, 1959) that the formation of squalene from MVA is dependent on the presence of –SH groups. It appears that the stereochemical approach might afford a clue for distinguishing between the two possibilities. Consider a molecule of MVA specifically labelled with deuterium at C-5 (Fig. 4). The configuration at this position will be retained in the formation of *iso*pentenyl pyrophosphate and dimethylallyl pyrophosphate. Direct displacement of the ester group of (IX) by the double bond of (VIII) should result in a net inversion of the cationic centre and afford a

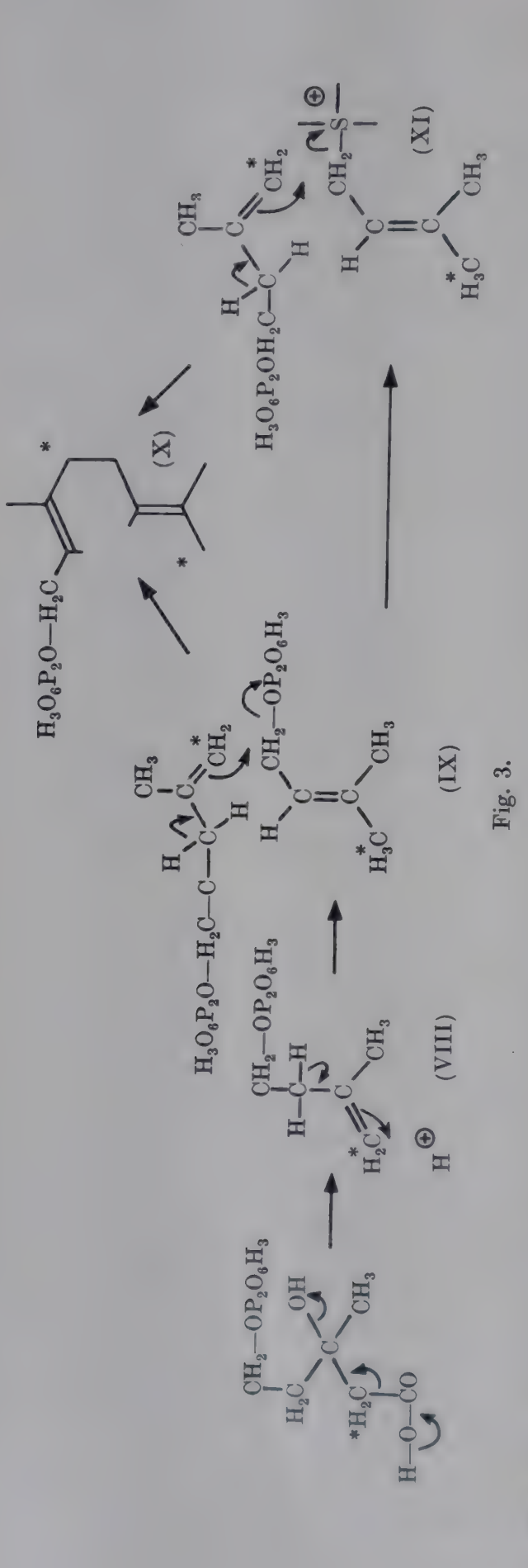
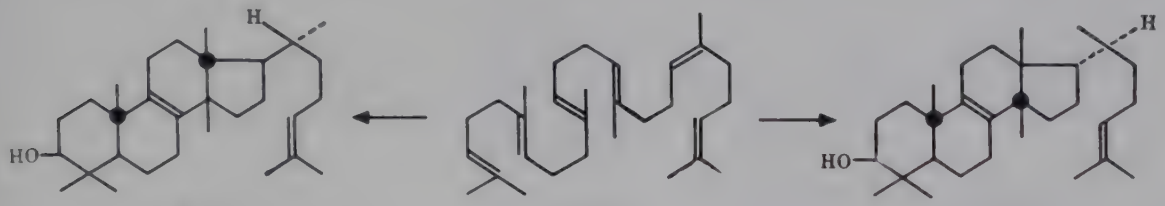


Fig. 3.

Fig. 4.

substance with the configuration shown in (XII). On the other hand, if the alkylation reaction is mediated by a sulphur-containing group (XI), then two such displacements should occur and the net result would be retention of configuration in the product (XIII). The problem could be settled by following the distribution of the deuterium atom. The known configuration at C-3 of MVA might be useful as an internal reference for the establishment of configuration at C-5, while the configuration of the product should be derivable, in principle, by a study of the axial or equatorial position of the deuterium atom in an appropriate cyclization derivative, e.g. (XIV).

A major stereochemical problem of terpene biosynthesis is linked with the formation of polycyclic derivatives from the aliphatic precursors. While the early biosynthetic theory (Ruzicka *et al.* 1953) emphasized mainly the structural relationships, the discovery and structural identification of diastereomeric substances like lanosterol (XV) and



(XV) (XVII) (XVI)

Fig. 5.

● indicates that the substituent at the ring junction is β, i.e. above the plane of the ring system.

tirucallol (XVI) made it clear that in the assumption of a single aliphatic precursor, squalene (XVII), steric factors had to be made responsible for the formation of different products. Two main aspects of the problem need be considered, namely the steric course of the cyclization reaction proper, and the steric course of the rearrangements which can be coupled with the cyclization.

As for the cyclization reaction, two main factors can be of influence: (*a*) the geometry of the participating double bonds; (*b*) the conformation of the cyclizing molecule. While the stereochemistry of the enzyme surface can hardly be discussed in the present lack of information, these two factors can be satisfactorily accounted for in an idealized scheme based upon a set of chemically reasonable assumptions (Eschenmoser, Ruzicka, Jeger & Arigoni, 1955; Ruzicka, 1956).

The influence of the geometry of the double bond is best illustrated in a model system representing a simple electrophilic addition (Fig. 6). If an acid $A^{\oplus}$ is added to a double bond with formation of a classical carbonium ion, the geometry of the system is lost and a non-specific subsequent addition of the base $B^{\ominus}$ must be taken into account. The

only way of preserving the original geometry lies in the assumption of cationic structures with a stable configuration, conveniently illustrated as bridged ions. Attack of the base $B^{\ominus}$ on such ions results in the production of a single isomer.

Such a mode of addition is referred to as antiplanar and is characterized by the planar position of the four centres involved. The scheme can be applied to the cyclization reaction if one keeps in mind that here the base is in fact represented by a second double bond. For the case of a cyclizing molecule (XVIII) the postulate of antiplanar addition poses

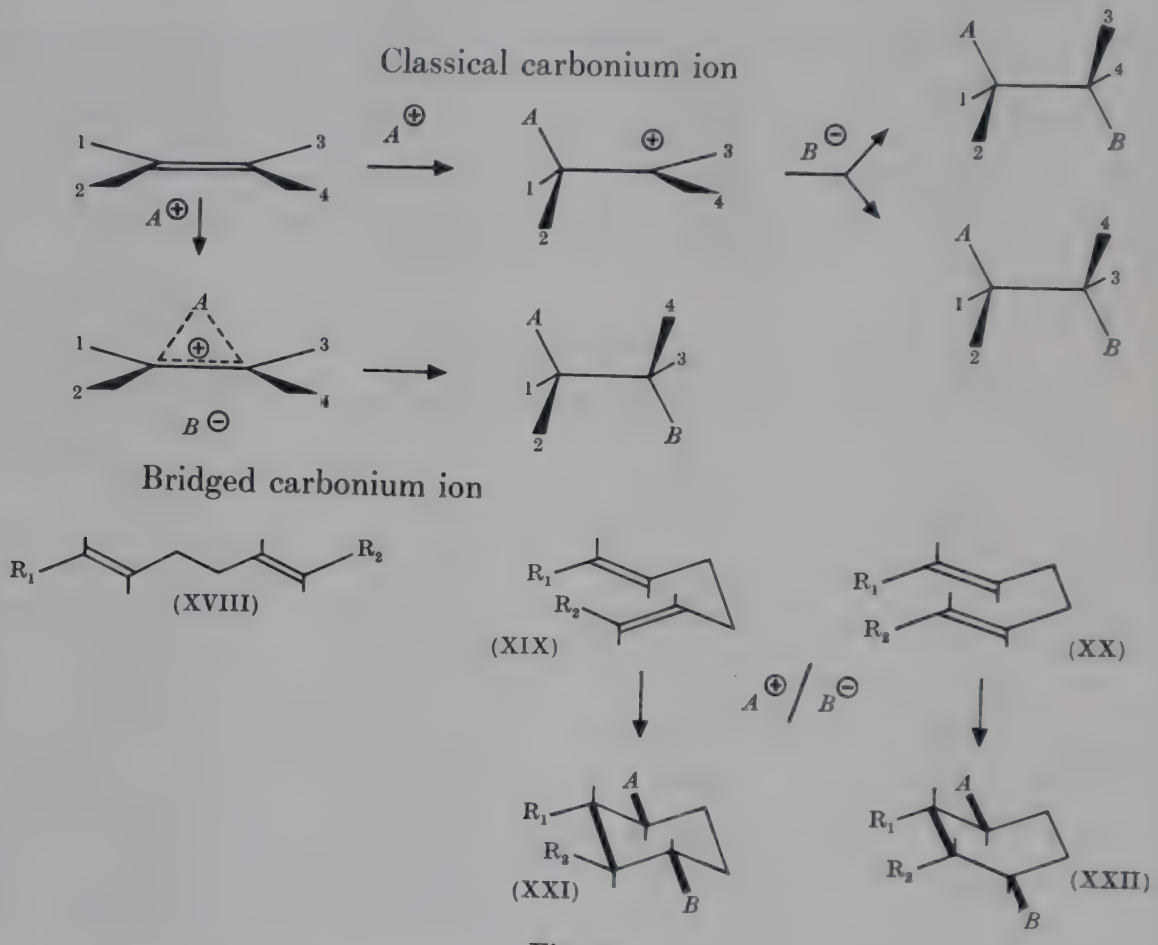


Fig. 6.

some restrictions to the conformational factor. It can be shown that among the many possible foldings of the chain only two fulfil the requirements of the postulate, namely the chair-type (XIX) and the boat-type folding (XX). Once the geometry of the double bond and the types of folding are fixed, the configuration of the products (XXI, XXII) can be derived unequivocally. On this basis the number of stereoisomers available is reduced to only two, from the possible total of eight. It must be underlined that in chemical (non-enzymic) experiments the energetically unfavourable boat-type folding will scarcely be used, whereas this need not be the case for the enzymic reaction, in which such a folding can be imposed by contact with the enzyme surface.

In a similar way it can be shown that, in a rearrangement of the type illustrated in Fig. 7, stereospecificity obtains if the rearrangement proper is formulated as a process in accord with the rules of antiplanar additions, in which the migrating group (R_2) is substituted for the base. The configuration of the product can again be deduced unequivocally from the geometry of the double bond.

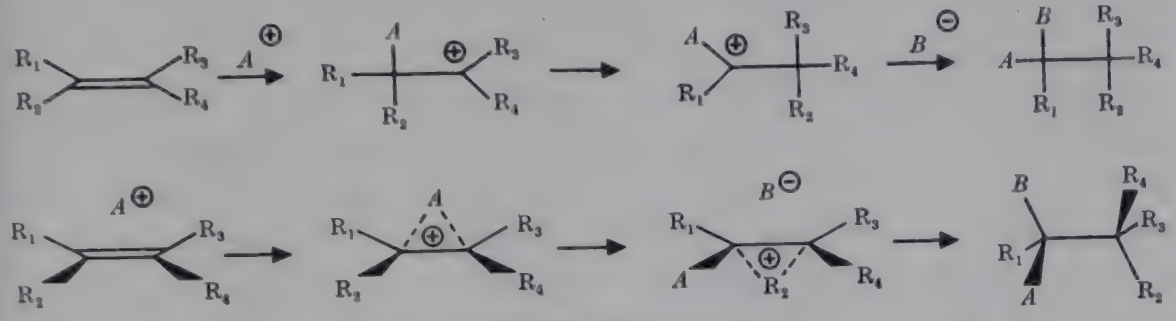


Fig. 7.

The rigorous application of these rules to the class of polycyclic triterpenes allows a stereochemical interpretation of their formation from a single all-*trans* squalene precursor, on the assumption that the reaction represents a 'non-stop' process initiated by the addition of a particle

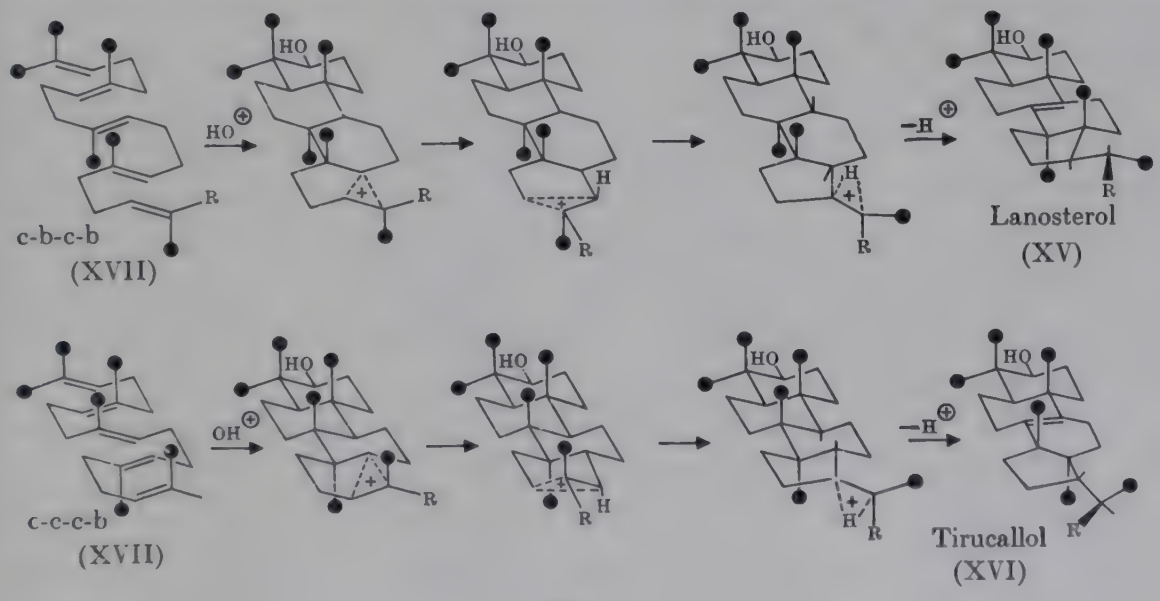


Fig. 8.

b, boat; *c*, chair.

$OH^{\oplus}$ (Eschenmoser *et al.* 1955). The only undefined element in the problem is the sequence of chair- and boat-type foldings, e.g. the exact conformation of the precursor, and this obviously must represent one of the main functions of the enzyme system. Once a given conformation has been 'frozen' by the enzyme, the detailed stereochemistry of the product can be derived by application of the rules. This is best illustrated by inspection of the two cyclization sequences of Fig. 8.

The formation of the two diastereomeric products lanosterol (XV)

and tirucallol (XVI) is clearly a consequence of the different conformation in which the second ring (*B*) is folded. The scope of this paper does not permit a detailed examination of all other polycyclic triterpenes (cf. Eschenmoser *et al.* 1955; Ruzicka, 1956). It is a rewarding fact that all representatives of the group investigated after the first proposal of the theory were found to fit the scheme without exception; no doubt in some cases the structural investigation was facilitated by the knowledge of the principles.

Recently experimental evidence has been adduced in support of the theory. In a brilliant piece of work two groups (Maugdal, Tchen & Bloch, 1958; Cornforth, Cornforth, Pelter, Horning & Popják, 1958) have independently shown that the biogenesis of lanosterol involves a double 1:2-shift of methyl groups as required by the scheme and not a single 1:3-shift—thus demonstrating the boat-type folding of ring *B* during the cyclization.

A further insight into the mechanism of the cyclization reaction has been gained during the study of the formation of pentacyclic terpenes in higher plants (Arigoni, 1958). After incorporation of [2-^{14}C]MVA into growing seedlings of soya beans, a mixture of labelled compounds was obtained, from which among other substances all-*trans* squalene and soyasapogenol D (XXIII) could be isolated in pure form. By degradation techniques it was shown that C-24 is specifically derived from the methyl group of MVA, and that all the label to be expected in the geminal substituents at C-4 is restricted to the equatorial methyl group. This must mean that in the precursor the two terminal methyl groups remain distinct throughout the condensation, and that therefore the isomerization of *iso*pentenyl pyrophosphate (VIII) to (IX) indeed represents a stereospecific process.

Furthermore it can now be specified that in the aliphatic precursor the label will be restricted to that one of the two carbon atoms which is *cis* to the hydrogen of the double bond, as is known to be the case for the internal isoprene units of the squalene chain (Cornforth, Cornforth, Popják & Gore, 1957). The fact that even during the cyclization no randomization of the isotope occurs and the observed distribution of radioactivity clearly show that ring *A* has been formed from a chair-type folding of the chain (cf. XXIV) and that at no time during the process the configurational stability of the cationic structure (XXV) has been lost. The general validity of the phenomenon has been verified experimentally with two diterpene mould metabolites, gibberellic acid (XXVI) (Birch, Richards & Smith, 1958*a*)* and rosenonolactone (XXVII)

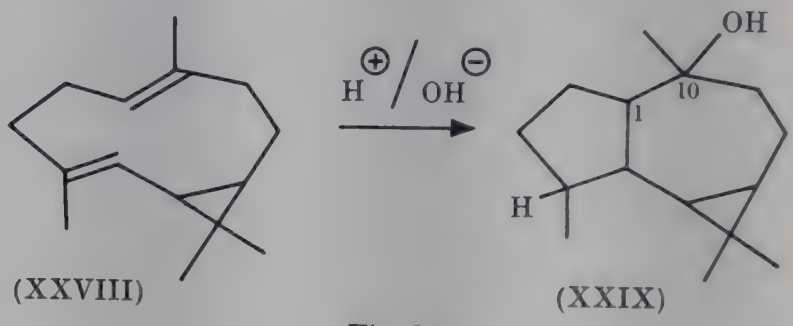
* For later references to structure and stereochemistry see Stork & Newman (1959), *J. Amer. chem. Soc.* **81**, 3168, 5518; Cross, Grove *et al.* (1959), *Proc. chem. Soc., Lond.*, p. 302; (1959), *Chem. & Ind.* p. 1345.

HO
R
(XXV)
HO
(XXIV)
OH
CO_2H
H
CO
O
HO
(XXVII)
(XXVI)
OCH_3
(XXIII)
CH_2OH
24
4
HO
CH_3
2
O
O

Fig. 9.

(Birch, Richards, Smith, Harris & Whalley, 1958*b*; Britt & Arigoni, 1958). The last-named substance furnished a striking example for the potentiality of the theoretical approach, since its biochemical investigation enabled the derivation of a stereochemical formula, which was eventually confirmed by chemical means.

The rules illustrated for the formation of polycyclic triterpenes should also apply in principle for the lower homologues.* No doubt this is true for many diterpenes and for some sesquiterpenes; however, it is observed that, as a rule, specialization of the enzyme systems involved and stereospecificity of formation decrease rapidly with the number of carbon atoms. One example will illustrate this point: the 10-membered bicyclic hydrocarbon (XXVIII) which might serve as a precursor of many sesquiterpenes can undergo cyclization in not less than four



(XXVIII) (XXIX)

Fig. 10.

different conformations and none of them can be excluded *a priori* on energy grounds for the enzymic reaction. It turned out recently (Dolejs, Herout, Motl, Šorm & Soucek, 1959; Birch, Grimshaw, Speake, Gascoigne & Hellyer, 1959) that Nature can produce four different compounds of the general formula (XXIX) differing only in the stereochemistry of C-1 and C-10; at least two of these cannot be derived in any direct manner from (XXVIII) through a process involving antiplanar addition. Clearly much new chemical and biochemical information must be collected before any useful generalization can be attempted.

An analogous situation holds for the problem of absolute configuration. Among the triterpenes and their steroidal derivatives high specialization of the enzyme system is a rule without exception, which always results in the production of the same enantiomeric type for the first two rings (*A* and *B*), as illustrated in the partial formula (XXX). This kind of specificity is lost in the diterpene field already, and many examples have been added by recent investigations to the list of those compounds which are characterized by an abnormal (sometimes called 'wrong') configuration at the *A*/*B* ring junction (for a summary, cf. Djerassi,

* *Note added in proof*: The extension of these rules to sesquiterpene biogenesis has been attempted in a recent paper by Hendrickson [Hendrickson, J. B. (1959). *Tetrahedron*, **7**, 82].

Cais & Mitscher, 1959). Kaurene, a tetracylic diterpene of as yet unknown structure (Briggs, Cawley, Loe & Taylor, 1950), represents a unique case, since it has been isolated from natural sources in both enantiomeric forms. Caution should therefore be applied to biogenetic arguments based exclusively on comparison of absolute configurations. Thus the fact that the hypothetical biogenetic precursor (XXXI) of eremophilone (XXXII) seems to possess the opposite absolute configuration to the majority of the compounds related to the eudesmol type of sesquiterpenes, e.g. eudesmol itself (XXXIII) (Djerassi, Riniker & Riniker, 1956), might lead us to suspect that the migration of the angular methyl group of (XXXI) is a direct consequence of its 'wrong' configuration. Such hypothesis, however, is strongly weakened by the existence of

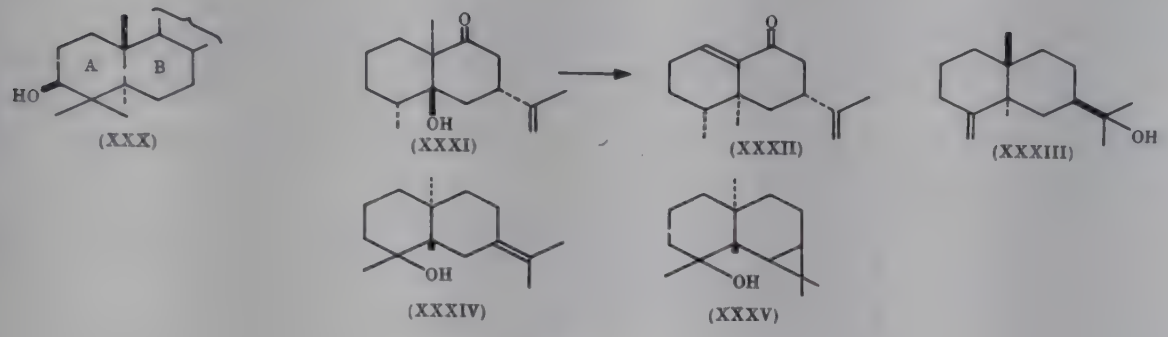


Fig. 11.

compounds like racemic juniper camphor (XXXIV) (Motl, Herout & Šorm, 1958) and maaliol (XXXV) (Büchi, Schach v. Wittenau & White, 1959) possessing both a 'wrong' configuration and a non-rearranged carbon skeleton*. On the other hand, the often quoted fact that the angular methyl group of eudesmol (XXXIII) and many related substances (Riniker *et al.* 1954; Klyne, 1953) occupy the same β-position as the methyl group at the ring junction of all known polycyclic triterpenes (cf. XXX) is nothing more than a fortuitous coincidence due to the arbitrary placing of the ring bearing the *iso*propyl group on the right side of the formula and can scarcely be of any biogenetic significance, since the two ring systems very probably owe their formation to different mechanisms.

Many stereochemical problems still await a solution in the field of terpene and steroid biosynthesis. It is hoped that the combined attack of chemists and biochemists will further contribute to a better understanding of this exciting chapter in the history of natural compounds.

* *Note added in proof*: According to the latest evidence [Zalkow, L. H., Markley, F. X. & Djerassi, C. (1959). *J. Amer. chem. Soc.* **81**, 2914] the absolute configuration of eremophilone is in fact enantiomeric with that shown in (XXXII).

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DISCUSSION OF PAPER BY ARIGONI

J. W. Cornforth (*National Institute for Medical Research, Mill Hill*): There is a point concerning the extent to which the very elegant elucidation of the absolute configuration of mevalonic acid can be taken to indicate the stereochemistry of the enzymic condensation of acetoacetyl-CoA with acetyl-CoA. This condensation is analogous to citrate synthesis from oxaloacetate and acetyl-CoA, in that a molecule of CoA is liberated. This liberation is usually explained as a hydrolysis following an initial aldol condensation; but citrate synthesis is reversible, and CoA-deacylases as a class do not catalyse resynthesis of the thiol ester group. It seems possible that two basic centres on the condensing enzyme may produce, in acetyl-CoA, electron shifts corresponding to the production of keten ($H_2C{:}CO$) from acetyl chloride and triethylamine, the activated species so produced combining with oxaloacetic acid, after the manner of keten with ketones, to give coenzyme A and the β-lactone of citric acid. β-Lactones are known to be hydrolysed abnormally by molecular water, the water molecule attacking the β-carbon and inverting its configuration. Thus any deduction from the configuration of mevalonic acid to the orientation of substrates on the condensing enzyme should take into account the possibility of an inversion at the asymmetric carbon atom.

STERIC ASPECTS OF DRUG ACTION

By R. B. BARLOW

Department of Pharmacology, University of Edinburgh

The present theories of the ways in which drugs may act date from the work of Overton and Meyer and of Ehrlich at the beginning of this century. Meyer (1899, 1901), Baum (1899) and Overton (1901, 1902) studied the effects of narcotics and observed a correlation between the potency of compounds and their distribution coefficients between olive oil and water. This led them to suppose that these narcotics affected cells by some physicochemical process. The ideas of Ehrlich (1913), based on his work with arsenical drugs, postulated the existence on the tissues of specific structures, called receptors, at which the drugs act.

The two theories are not mutually exclusive. In certain circumstances potency may be related to physicochemical properties, whereas in others an action at a receptor is more likely. Clark (1933) measured the concentrations of certain compounds which produced detectable effects on frog-heart muscle and calculated the percentage of the area of the cells which might be covered with drug molecules in these concentrations. He found that ouabain, acetylcholine, atropine, adrenaline and histamine could not possibly cover more than a fraction of the area of the cells, whereas caffeine and the aliphatic alcohols (in the range heptyl–dodecyl) had to be present in amounts which would be sufficient for them to form a monomolecular layer over the whole area of the cells.

When the action of a drug depends on its physicochemical properties, the size and shape of the molecule should be important only in so far as they affect these. In consequence, this type of action is characterized by the absence of any critical relationship between size and shape, and activity. In most problems the actions of drugs appear to involve a receptor mechanism and here the size and shape of the molecule is extremely important in determining the fit of the drug to the receptor.

It is possible to treat the receptor entirely as a hypothetical structure, which gives rise to the biological response when a complex is formed with a drug. This attitude has been recommended by Stephenson (1956) because it avoids making unrecognized and unjustified assumptions about the properties of receptors. Nevertheless, although there is usually no justification for it, it is possible to think of the receptor as being the active spot on an enzyme and that combination of the drug with the receptor, or rather of particular pharmacodynamic groups in the drug with particular groups in the receptor unit (Fig. 1), leads to a sequence of reactions which culminate in the biological response.

In either situation, whether the receptor is regarded as a hypothetical entity or as the active spot on a hypothetical enzyme, two factors will determine the activity of a drug. These have been called (Stephenson, 1956) the affinity (or adsorbability) and the efficacy (or the ability of the compound, when adsorbed, to set off the sequence of reactions). Clark & Raventos (1937) had concluded that equiactive doses of stimulant drugs produced an equal number of drug–receptor complexes, but Stephenson (1956) has shown that this is not generally true. A small number of complexes with a compound of high efficacy may produce the same effect as a large number of complexes with a compound of low efficacy.

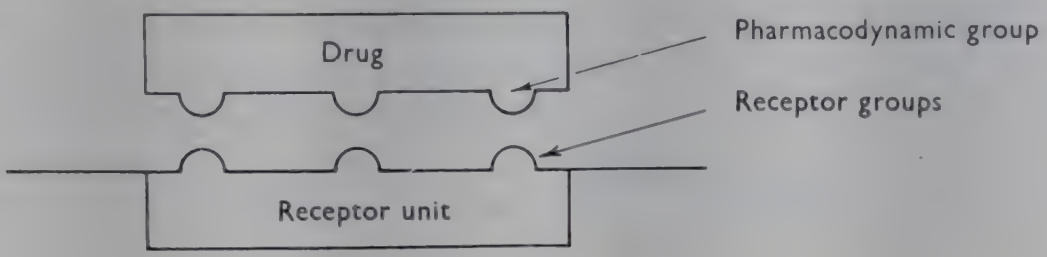


Fig. 1. Formation of a drug–receptor complex.

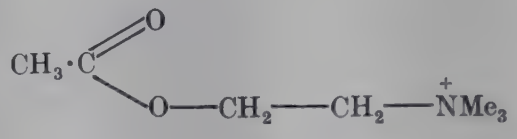
With substances which produce a positive response (such as the contraction of a muscle fibre caused by acetylcholine) it is difficult to assess the separate contributions to activity of efficacy and affinity. Substances which antagonize the action of stimulant compounds (as atropine antagonizes the action of acetylcholine) have, however, no efficacy, and antagonist potency depends, therefore, solely on affinity. It is conceivable that steric factors may play some part in determining efficacy, because this may depend upon the possession by the drug of a particular group in a position accessible to the enzyme. The effects of steric factors on affinity, however, should also be very great and should, by the study of antagonists, be more easily assessed. With stimulant compounds, however, it is seldom possible, on the existing evidence, to decide how far changes in structure affect efficacy and how far they affect affinity.

Three steric factors must be considered: (1) the arrangement in space of the pharmacodynamic groups of the drug; (2) the arrangement in space of the receptor groups in the receptor unit; (3) the arrangement in space (on the biological surface) of the receptor units themselves.

Although our knowledge of the first factor has benefited greatly from advances in the determination of absolute stereochemical configurations and in assessment of the most likely conformations of flexible molecules, knowledge of the arrangement in space of the receptor groups in the receptor unit is still, inevitably, obtained by

arguing backwards from the structures of active compounds and their antagonists. Information about the enzymes, of which the receptors may be the active spots, is still almost entirely non-existent. The existence of the third factor has become recognized in the last ten years and it is discussed towards the end of this paper.

In recent years the absolute stereochemical configuration of a number of pharmacologically important substances has been elucidated. Among the most important developments have been the assignment of an absolute configuration to morphine (Kalvoda, Buchschacher & Jeger, 1955; Bentley & Cardwell, 1955; Mackay & Hodgkin, 1955), to certain synthetic analgesics such as Methadone, Phenadoxone and Betaprodine (Beckett & Casy, 1955, 1957; Beckett & Harper, 1957; Beckett, Casy, Kirk & Walker, 1957), and to adrenaline and other sympathomimetic substances (Drell, 1955; Pratesi, La Manna, Campiglio & Ghislandi, 1958). These have recently been reviewed by Beckett (1959). The present paper is concerned with developments among substances which imitate or antagonize the actions of acetylcholine (I).



(I)

Acetylcholine is the chemical transmitter of nerve impulses at the neuromuscular junction in the voluntary nervous system, and at ganglia and post-ganglionic parasympathetic synapses in the autonomic nervous system. The actions of acetylcholine at the neuromuscular junction and at ganglia are imitated by small amounts of nicotine and have been called the 'nicotine-like' actions of acetylcholine. The actions at post-ganglionic parasympathetic synapses are imitated by muscarine and have been called the 'muscarine-like' actions of acetylcholine. Many substances act like acetylcholine, but most of them differ from it in that their actions are predominantly either nicotine-like or muscarine-like. Many substances which have, like acetylcholine, both high nicotine-like and high muscarine-like activity, have been shown to act, not at the receptor units affected by acetylcholine, but by preventing the destruction of acetylcholine by the cholinesterases which normally limit its action.

Although the actions of acetylcholine at the neuromuscular junction and at ganglia are classified as nicotine-like, the receptors in these structures are not identical. They differ also from one type of neuromuscular junction to another, and from one animal species to another. Differences are likewise found between the receptor units in different

sorts of ganglia. The difference between the receptors in the ganglia and those in the neuromuscular junction is well illustrated by the variation of antagonist activity with structure in the polymethylenebistrimethylammonium salts: ganglion-blocking activity is maximal in the hexamethylene compound, Hexamethonium, but neuromuscular blocking activity is maximal in the decamethylene compound, Decamethonium (Table 1; Paton & Zaimis, 1949). Nevertheless, in spite of the differences between the receptors at the various sites of the nicotine-like actions of acetylcholine, it is convenient to group them together to distinguish them from the receptors in the sites of the muscarine-like actions at the post-ganglionic parasympathetic synapses.

Table 1. *Relative blocking activity of polymethylenebistrimethylammonium* ($Me_3\overset{+}{N}\cdot[CH_2]_n\cdot\overset{+}{N}Me_3$) *iodides* (*from Paton & Zaimis*, 1949)

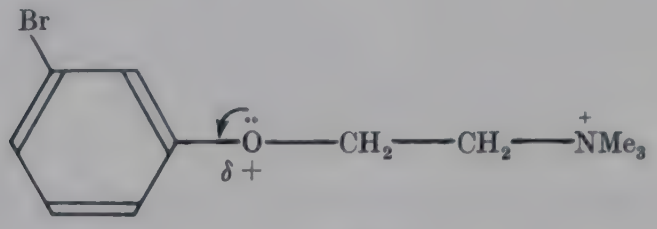
At ganglia (cat's superior cervical ganglion)		At the neuromuscular junction (cat's sciatic nerve-tibialis preparation)	
n	Relative activity†	n	Relative activity†
4	2	7	1
5	78	8	18
6	100*	9	81
7	10	10	100*
8	2	11	51
		12	32

* Arbitrary standard.
† Molar basis.

The early work on the structural features associated with nicotine-like and muscarine-like activity has been reviewed by Barlow (1955*a*) and is only briefly discussed here. For nicotine-like activity, i.e. for activity at the neuromuscular junction and in ganglia, it seems that a molecule should contain a cationic head of a suitable size, e.g. a nitrogen atom with at least two methyl groups, $> \overset{+}{N}(Me)_2$, and a partial positive charge in a position roughly corresponding to that of the ether oxygen of acetylcholine. Most of the substances with high nicotine-like activity contain these groups, including the substituted phenyl ethers of choline described by Hey (1952), some of which, e.g. the *m*-bromophenyl ether (II), are more than 100 times as active as acetylcholine.

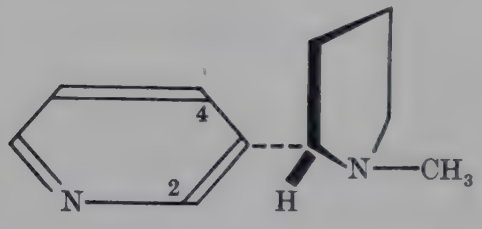
Compared with these ethers, nicotine itself is a fairly rigid structure. Taylor (1951) has shown that, at body pH, nicotine exists as the nicotinium ion with the proton on the pyrrolidine nitrogen. The absolute configuration of nicotine was worked out by Hudson & Neuberger (1950) and the natural (−)-alkaloid has the configuration (III) which may be called L- according to the standard amino acid system or *S*

according to the system of Cahn, Ingold & Prelog (1956) (see introductory note on p. vi). The relative positions of the cationic head and the pyridine ring can be the same in both isomers, which can then be regarded as differing only in the relative positions of the pyrrolidine ring. If there are only two pharmacodynamic groups, the cationic head and the carbon atoms in the 2 or 4 position of the pyridine ring which carry a partial positive change, it might be expected that the *R* and *S*



(II)

forms would have the same activity. The two have not been compared on isolated tissue preparations, but Hicks, Brücke & Hueber (1935) found no significant differences between the activity of (+)- and (−)-nornicotine on the blood pressure, respiration, vagal and sympathetic ganglia, and the heart of cats. There were slight differences in the activity on frog muscle and nerve–muscle preparations. Hicks & Sinclair (1947) found the toxicities of *R*- and *S*-nicotine to be identical



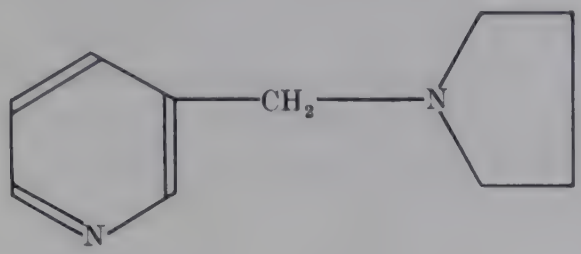
L-Nicotine (*S*-configuration)

(III)

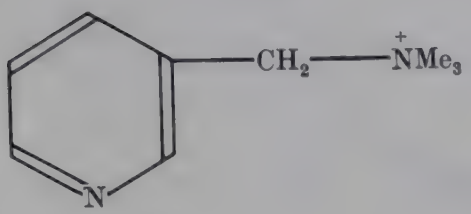
in guinea pigs. The insignificance of the pyrrolidine ring is emphasized by preliminary tests (R. B. Barlow & J. T. Hamilton, unpublished work) of an isomer of nicotine, *N*-(*β*-pyridylmethyl)pyrrolidine (IV). On the frog rectus and rat blood pressure this substance is about as active as *S*-nicotine. The corresponding trimethylammonium compound, trimethyl-(*β*-pyridylmethyl)ammonium (V), appears to be ten or more times as active.

For muscarine-like activity it seems that a molecule should contain a cationic head with two methyl groups, a polar group in a position about the same as that of the ester link in acetylcholine, and an overall length not greater than that of trimethyl-*n*-pentylammonium. This last factor

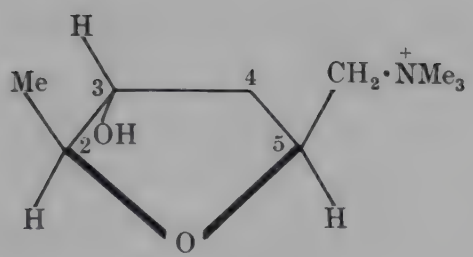
was suggested by Ing (1949) and has been called the 5-atom chain rule. In recent years the most striking development has been the work of two groups of investigators, who have established the absolute configuration of muscarine (VI *a*; the affix *b* used subsequently in the text indicates the mirror image) and its isomers (VII *a*, VIII *a*, IX *a*), and synthesized



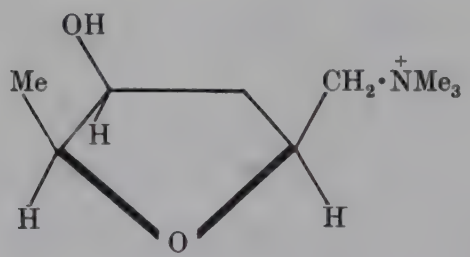
(IV)



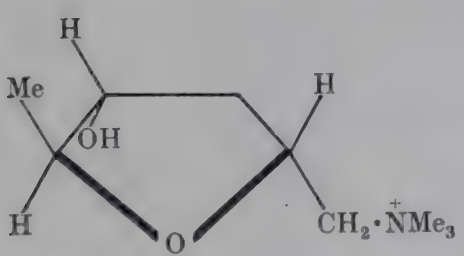
(V)



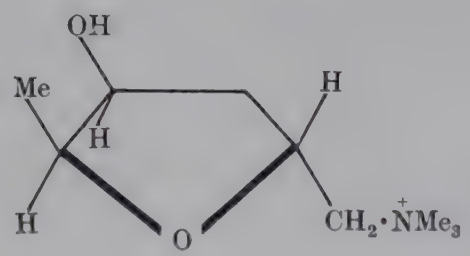
Muscarine 2*S*:3*R*:5*S*
(VI*a*)



*epi*Muscarine 2*S*:3*S*:5*S*
(VII*a*)



*allo*Muscarine 2*S*:3*R*:5*R*
(VIII*a*)



*epiallo*Muscarine 2*S*:3*S*:5*R*
(IX*a*)

them all (Eugster & Waser, 1954; Eugster, 1956*a*, *b*; Eugster, 1957*a*, *b*; Eugster & Waser, 1957; Eugster, Häfliger, Denss & Girod, 1958*a*, *b*, *c*, *d*; Kögl, Salemink, Schouten & Jellinek, 1957; Kögl, Cox & Salemink, 1957*a*, *b*; Corrodi, Hardegger, Kögl & Zeller, 1957; Hardegger & Lohse, 1957; Corrodi, Hardegger & Kögl, 1957; Cox *et al.* 1958; Hardegger, Furter & Kiss, 1958). Natural (+)-muscarine has the 2*S*:3*R*:5*S*-

configuration. It is interesting that, as is readily seen on models, this is a rather inflexible structure. The hydrogen atom at C-5 restricts the rotation of the trimethylammonium group and it seems likely that the atoms cannot depart greatly from the arrangement shown in Fig. 2.

Estimates of the activity of the isomers of muscarine have been made by Waser (1958) and by Gyermek & Unna (1958). These show (Table 2) that (−)-muscarine (2*R*:3*S*:5*R*; VI *b*) is only feebly active and suggest that the relative positions of three groups in the molecule—the quaternary nitrogen, the alcoholic hydroxyl and probably the ether oxygen—are extremely important for pharmacological activity. In *epi*muscarine

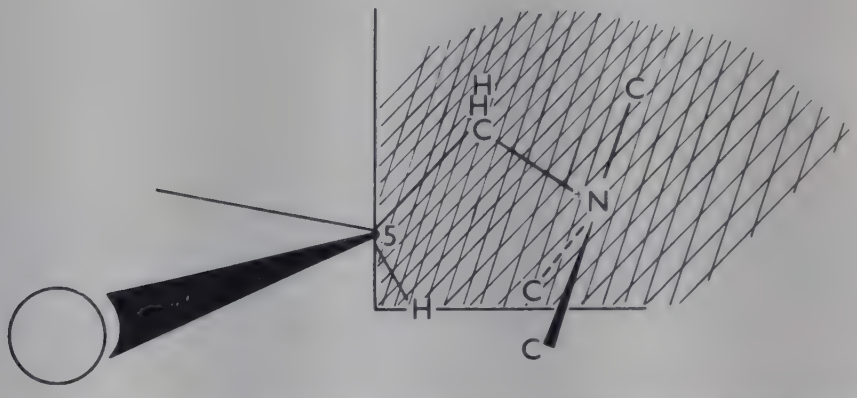


Fig. 2. Restriction of rotation of trimethylammonium group by the 5-hydrogen atom in muscarine.

Table 2. *Relative activity of compounds related to muscarine*

The equipotent molar ratios are defined as the number of molecules of the compound required to produce the same effect as one molecule of (+)-muscarine iodide (or chloride): a high figure indicates low activity.

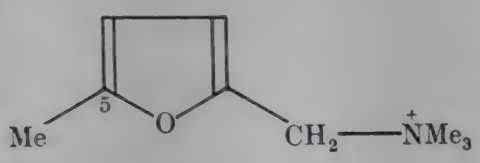
If one isomer were completely inactive, the active isomer should be twice as powerful as the racemate. The values marked * indicate that the active isomer is more than twice as active as the racemate and imply that the inactive isomer must somehow be acting as an antagonist of the active isomer.

Figures of activity on the frog heart and cat blood pressure have been recalculated from the results of Waser (1958); the other figures are taken from Gyermek & Unna (1958).

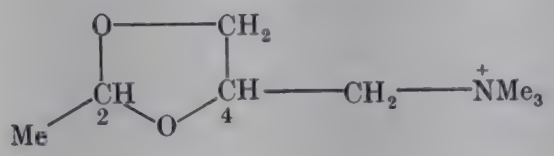
		Equipotent molar ratios				
Compound	Salt	Frog heart	Cat blood pressure	Dog bladder	Rabbit ileum	Mouse miosis
(+)-Muscarine	I, Cl	1·0	1·0	1·0	1·0	1·0
(±)-Muscarine	I	1·78	2·5*	2·8*	3·1*	1·6
(−)-Muscarine	Cl			800	400	200
(±)-Normuscarine	Cl	429	1925			
(±)-*epi*Muscarine	I	11 900	750	1700	700	600
(±)-*allo*Muscarine	I	833	420	500	460	600
(±)-*epiallo*Muscarine	I	667	250	365	660	140
(+)-Muscarone	I			0·52	0·45	0·32
(±)-Muscarone	I	0·56	0·37	0·35	0·4	0·2
(−)-Muscarone	I	0·42	0·25	0·17	0·19	0·19
(±)-*allo*Muscarone	I	1·1	0·75	0·77	0·84	0·97
(±)-4:5-Dehydromuscarone	Cl	0·56	0·37	1·62	1·5	3·0
Acetylcholine	Cl	0·18	4·2	—	—	—

(VII *a*, *b*) the quaternary nitrogen and the alcoholic hydroxyl groups are on the same side of the ring, and it might therefore be expected that the optical isomers of this structure and of *allo*muscarine (VIII *a*, *b*) would be inactive.

In the 2*R*:3*R*:5*S*-form of *epiallo*muscarine (IX *b*), however, the quaternary nitrogen atom and the alcoholic hydroxyl group are in the same relative positions with respect to the ring as they are in (+)-muscarine (VI *a*); the only difference is in the position of the 2-methyl group. (±)-*epiallo*Muscarine, however, though more potent than (±)-*epi*muscarine and (±)-*allo*muscarine, is only feebly active. This suggests that when the 2-methyl group is *cis* to the 3-hydroxyl group it destroys the fit of the molecule to the receptor groups. It may do this by restricting the rotation of the 3-hydroxyl group, preventing it from becoming attached to the appropriate receptor group (this would be consistent with the very low activity of *epi*muscarine), or it may do so



(X)



(XI)

simply because of steric effects, a large methyl group projecting below the ring preventing a close fit between the molecule and the receptor groups.

On models the 2-methyl group does not appear to project much further from the ring than does the 3-hydroxyl group, and there is evidence that it may itself be an additional point of attachment to a receptor group. Ing, Kordik & Williams (1952) found that the muscarine-like activity of 5-methylfurfuryltrimethylammonium (X) was between 10 and 20 times that of furfuryltrimethylammonium. Also 2-methyl-4-trimethylaminomethyl-1:3-dioxolan, compound F 2268 (XI), is very much more active than the corresponding compound without a methyl group in this position (2 in F 2268 and muscarine, 5 in the furfuryl compound; Fourneau, Bovet, Bovet & Montezin, 1944). The 5-methyl group in 5-methylfurfuryltrimethylammonium (X), however, is in the plane of the ring and in F 2268 it may be either above or below (it is not known which of the four isomeric forms of this compound is the most active). It is possible to suppose that there is a receptor group with which the 2-methyl group interacts, so placed, with respect to the receptor group with which the 3-hydroxyl group interacts, that compounds are highly active when the two groups are *trans*. Instead it may be supposed that the two receptors are much closer together so that the

cis position would fit better, but that a 3-hydroxyl group is sterically hindered by a *cis*-2-methyl group. Yet another possibility is that there is no receptor which interacts with a 2-methyl group, and that the effect on activity is caused by its inductive effects on the nearby oxygen atom, as well as by steric hindrance of a *cis*-3-hydroxyl group.

The high activity of the muscarones (compounds with carbonyl at C-3) is remarkable, particularly as the various isomers do not differ greatly in potency. In the most active compound, (−)-muscarone, the 2-methyl group and the 5-methylenetrimethylammonium group are *cis* as in muscarine, but there is no indication whether the (−)-isomer is related to (−)-muscarine or to (+)-muscarine. The lack of stereospecificity in the muscarones suggests virtually a two-point attachment. The force binding the negatively-charged carbonyl-oxygen atom to the receptor would seem to be greater than those binding the negatively-charged hydroxyl-oxygen atom in muscarine and any others involving the 2-methyl group or the ether oxygen atom in the ring, or both. It is therefore puzzling that the removal of the 2-methyl group in muscarone, as in muscarine, greatly decreases pharmacological activity (Zwicky, Waser & Eugster, 1959).

In this discussion differences in activity have been taken to indicate differences in affinity, but the results in Table 2 do not strictly justify this. If only one isomer is active in a racemic mixture, this isomer should be twice as active as the racemate, but on some preparations (+)-muscarine appears to be more than twice as active as (±)-muscarine. This implies that the (−)-isomer is an antagonist of the (+)-isomer and it must therefore have affinity. This point is obviously important, and it would be interesting to have figures for the activity of at least one of the resolved forms of each of the other isomers—*epi*muscarine, *allo*-muscarine and *epiallo*muscarine.

The structure of muscarine and the activity of the muscarones is particularly interesting in view of the high activity of F2268 (XI), which has been estimated as being 100 times that of acetylcholine (Salle & Valade, 1954). In this compound the two oxygen atoms can be regarded as equivalent. There are two asymmetric centres, but the compound has not been resolved; by analogy with the muscarones it might be expected that the various isomers would not differ greatly in activity.

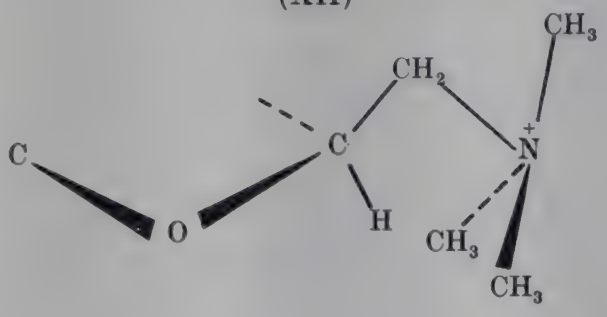
Acetyl-β-methylcholine (2-acetoxypropyltrimethylammonium; XII) is another muscarine-like compound which is optically active. The (+)-isomer is reported to be about as active as acetylcholine and about 200 times as active as the (−)-isomer on isolated intestine (Major & Cline, 1935; Simonart, 1938). On models it appears that the ether oxygen and the quaternary ammonium group can assume the same

relative positions as in muscarine (Fig. 2) in the *S*-isomer but not in the *R*-isomer because of steric interference by the β-methyl group. By analogy with muscarine, therefore, the active (+)-isomer ought to have the *S*-configuration.*

The foregoing discussion has been concerned mainly with rigid or sterically hindered molecules. It is much more difficult to assess the importance of steric factors in flexible molecules, but this has been attempted. Schueler (1953*a*) measured on models the maximum and minimum possible distances between the quaternary nitrogen atom and the carbonyl and ether oxygen atoms of muscarine-like substances.

$CH_3 \cdot CO \cdot O \cdot CHMe \cdot CH_2 \cdot \overset{+}{N}Me_3$

(XII)



(*S*-enantiomer)

He compared these values with the pharmacological activity, but this was not particularly informative, partly because he was working from the old incorrect formula of muscarine (Kögl, Veldstra & Van der Laan, 1942). Schueler also calculated the probability of two pharmacodynamic groups' being a particular distance apart in a flexible molecule, assuming free rotation of the C–C bonds (the calculations were based on the work of Guth & Mark, 1934). He made similar calculations and assumptions for two receptor groups in a receptor unit and discussed the various situations which might arise. Fig. 3, for instance, shows the distributions for two pharmacodynamic groups in a drug and two groups in a receptor unit when the mean distance between the receptor groups ($\mu_{L,\,R}$) is greater than the mean distance between the two pharmacodynamic groups ($\mu_{L,\,D}$). The area of overlap (shaded) gives an estimate of the adsorbability (affinity) of the drug.

In a subsequent paper Schueler (1953*b*) applied these ideas to assess the probability of two quaternary ammonium groups in a polymethylenebisquaternary ammonium salt being a particular distance apart, so that they might fit on to two receptor units at once. Here it was

* *Note added in proof*: This has actually been shown to be correct [Ellenbroek, B. W. J. & van Rossum, J. M. (1960). *Arch. int. Pharmacodyn.* (in the Press).] Thanks are given to Professor E. J. Ariens for advance notice of this result.

necessary to consider, in addition to the 'statistical effect' caused by the rotation of the various bonds, the mutual repulsion of the two onium groups. This destroyed the symmetry of the distribution curve, displacing it towards longer values. Schueler concluded, however, that only in short molecules did the average length approach the maximum extended length.

To test the validity of his ideas, Schueler studied the adsorption of polymethylenebistrimethylammonium salts on the synthetic resin Dowex 50. There was a rise in adsorbability from the ethylene compound to the trimethylene, followed by a decline, but the results did not really resemble the variation of either ganglion-blocking or neuro-

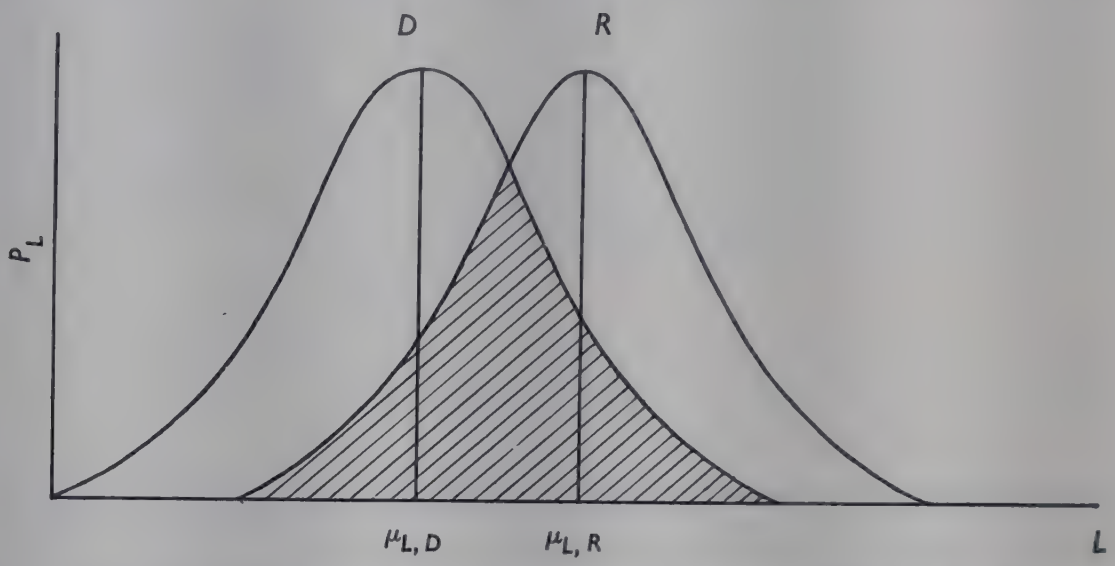


Fig. 3. Distribution curves showing the probability (P_L) of two groups in the drug (D) and in the receptor (R) being a particular distance (L) apart. $\mu_{L,D}$ and $\mu_{L,R}$ are mean differences between the two pharmacodynamic groups and the two receptor groups respectively.

muscular blocking activity with chain length. The distance between the receptor units of the resin was clearly too short for it to be used as a satisfactory model of the biological surface at which the compounds act.

Gill (1959) has pointed out certain errors in Schueler's procedure, particularly in assuming free rotation of the C–C bonds. In *n*-butane, for instance, thermodynamic factors produce a potential barrier, with a maximum value of about 4 kcal./mole, hindering free rotation about the bond C-2–C-3. The most likely conformation is with the two largest groups *trans* (Fig. 4). The next most likely are the skew positions and the relative probabilities *trans*:*skew*:*skew* are 1·0:0·27:0·27. Gill calculated the interquaternary distance/probability distributions for a number of compounds and compared the areas of the distribution diagram lying between the distances of 6·0 and 7·8 Å with the ganglion-blocking activity of the compounds. Some of the results are shown in Table 3. They strongly suggest that both ends of the active compounds

become attached to receptor units in ganglia but, on pharmacological evidence, Ing & Gill (1958) suppose that these receptor units are dissimilar. One is presumed to be an 'acetylcholine receptor' and the other an 'anchoring site', which is of different size, shape and function.

The possibility that a drug may combine with two receptor units at once was suggested as a possible explanation for the high neuromuscular blocking activity of decamethonium (Barlow & Ing, 1948).

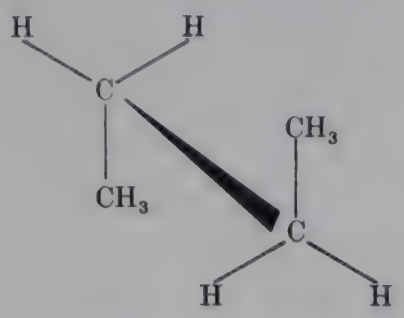


Fig. 4. Most probable conformation of *n*-butane.

Although, when regarded strictly as a hypothetical entity, receptor units might be regarded as distributed in a random fashion on the biological surface, it is possible that they may in fact be distributed in a regular pattern. They may, for example, be groups which are part of

Table 3. *Ganglion-blocking activity of bistrimethylammonium salts*

A = Proportion of the area of the distribution curve lying between 6·0 and 7·8 Å.
B = Activity in blocking transmission in the superior cervical ganglion of the cat (Gill, 1959).

	n	Calc. area (*A*)	Relative molar activity (*B*)
$Me_3\overset{+}{N}\cdot[CH_2]_n\cdot\overset{+}{N}Me_3$	4	5	1
	5	100	63
	6	62	100
	7	25	12
	8	18	3
$Me_3\overset{+}{N}\cdot p\text{-}C_6H_4\cdot[CH_2]_n\cdot\overset{+}{N}Me_3$	1	0	0
	2	100	100
	3	30	9
	4	18	2

particular amino acid units and recur in a particular sequence in a protein structure. A drug which can combine with two receptor units at once (this has been called being 'pharmacologically bivalent') should have very high affinity for two reasons: (1) When one end of the molecule dissociates, the other end is likely still to be tethered to the biological surface, and so the dissociated end will be held in a position ready for readsorption. (2) There may be considerable attraction between the molecule and the biological surface (other than at the

receptor groups) by van der Waals forces between the two, if they can fit closely together.

Table 4. *Relative neuromuscular blocking activity of choline esters of aliphatic dicarboxylic acids in rabbits (by the head-drop test)*

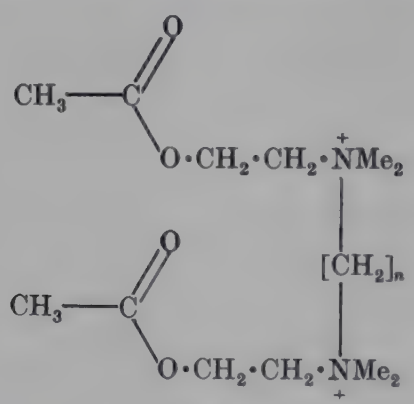
$$Me_3\overset{+}{N}\cdot CH_2\cdot CH_2\cdot O\cdot CO\cdot [CH_2]_n\cdot CO\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}Me_3$$

The results are recalculated from Bovet, Bovet-Nitti, Guarino, Longo & Fusco (1951). The actual head-drop dose of suxamethonium was 0·2 mg./kg.

n	Relative potency
0	0·027
1	1·0
2 (suxamethonium)	10·0
3	4·0
4	4·0
5	0·67*
8	0·4*

* In doses which caused convulsions.

The idea that decamethonium may be pharmacologically bivalent receives support from the variation of neuromuscular blocking activity with structure among choline esters of aliphatic dicarboxylic acids. The most active compound is the succinyl ester, suxamethonium (Bovet, Bovet-Nitti, Guarino, Longo & Marotta, 1949; Fusco, Palazzo,

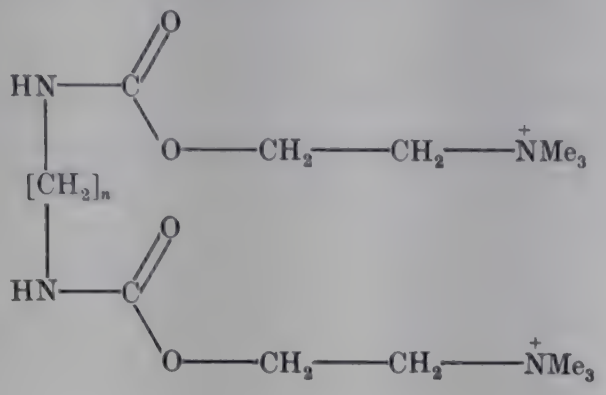


(XIII)

Chiavarelli & Bovet, 1949; Bovet, Bovet-Nitti, Guarino, Longo & Fusco, 1951; Table 4).

Further evidence has been obtained from the study of polymethylene-bisacetoxyethyldimethylammonium salts (XIII), which may be regarded as being two molecules of acetylcholine linked through a methyl group on the quaternary nitrogen (Barlow, 1955*b*). On the frog rectus, frog heart and cat blood pressure there is a sharp maximum in activity (Table 5) at the decamethylene compound. These compounds are acetylcholine-like, but the decamethylene compound is less active than

acetylcholine. This is presumed to be because the polymethylene chain attached to the quaternary nitrogen has reduced the efficacy. This change in efficacy should be about the same for all the compounds, so changes in activity should reflect changes in affinity. The results, therefore, have been taken to indicate that the affinity of the decamethylene compound is very much higher than that of the nona-



(XIV)

methylene compound and are in accord with the idea that the decamethylene compound is pharmacologically bivalent on these preparations. The decline in affinity above the decamethylene compound could be explained by the need for the polymethylene chain to bend in order for the two onium groups to fit the receptors.

Table 5. *Activity of polymethylenebisacetoxyethyldimethylammonium salts on the frog rectus, frog heart and cat blood pressure*

$CH_3 \cdot CO \cdot O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}Me_2 \cdot [CH_2]_n \cdot \overset{+}{N}Me_2 \cdot CH_2 \cdot CH_2 \cdot O \cdot CO \cdot CH_3$

The equipotent molar ratio shows the number of molecules of the compounds (±S.E.) required to produce effects comparable with those of one molecule of acetylcholine. A high number indicates low activity. Each value for the frog rectus and frog heart is the mean of three experiments; those for the cat blood pressure are the results of one experiment only (Barlow, 1955*b*).

	Equipotent molar ratios		
n	Frog rectus	Frog heart	Cat blood pressure
9	4317±438	1760	704
10	13·3±0·5	26·3±4·1	34·5
11	156±21	198±27	472
12	142±26	158±31	463

Somewhat similar compounds (XIV), in which two carbamylcholine molecules have been linked by a polymethylene chain attached at the carbamyl nitrogen, have been studied by Klupp, Kraupp, Stormann & Stumpf (1953), Kraupp, Klupp, Stormann & Stumpf (1954) and Cheymol, Delaby, Chabrier, Najer & Bourillet (1954). In these, neuromuscular blocking activity is maximal in the hexamethylene compound

(Table 6). There is also a marked rise—about tenfold—in acetylcholine-like activity on the frog rectus and guinea-pig ileum in proceeding from the tetramethylene compound to the hexamethylene (figures for the pentamethylene compound were not obtained on these preparations).

Table 6. *Relative neuromuscular blocking activity of choline esters of polymethylenebiscarbamic acids in rabbits (by the head-drop test)*

$$Me_3\overset{+}{N}\cdot CH_2\cdot CH_2\cdot O\cdot CO\cdot NH\cdot [CH_2]_n\cdot NH\cdot CO\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}Me_3$$

The results are recalculated from Cheymol, Delaby, Chabrier, Najer & Bourillet (1954). The actual head-drop dose of the compound with $n = 6$ was 0·034 mg./kg.

n	Relative potency	n	Relative potency
0	0·01	5	2·4
1	0·51	6	10·0
2	0·29	7	11·7*
3	0·41	10	3·8
4	1·1		

* This compound is possibly more active than the hexamethylene compound, but was not tested by the other group of workers.

The position of the maximum in this series is very puzzling. The neuromuscular block produced by the hexamethylene compound has the same pharmacological characteristics as those produced by decamethonium (Stumpf & Kraupp, 1956); it seems unlikely, nevertheless, that this compound is combining with two sets of receptors in exactly the same way as decamethonium may do. It is difficult to judge how far apart the two onium groups will be in such a molecule, but there is no reason to suppose that the distance should be greatly different from what it is in the choline esters of aliphatic dicarboxylic acids. It is possible that the receptor units are distributed differently in different directions in space and that the hexamethylenebiscarbamyl compound can combine with two units in either of the ways A and B shown in Fig. 5. If this were so, there should be a similar rise in the activity of other polymethylenebisonium compounds when the distance between onium groups becomes the same as in the hexamethylenebiscarbamyl compound. Although the complete series of compounds has not been studied, there does not seem to be any sign of this in the series of choline esters of aliphatic dicarboxylic acids (Table 4). In the polymethylene-bistrimethylammonium series the compounds intermediate between tri-decamethylene and octadecamethylene have not been tested, but in the analogous bistriethylammonium compounds both neuromuscular blocking and ganglion-blocking activity rise steadily up the series, without any marked rise at the decamethylene compound. Ganglion-blocking and neuromuscular blocking activity appear to be maximal at

the pentadecamethylene and hexadecamethylene compounds (R. B. Barlow & J. R. Vane, unpublished work; R. B. Barlow & J. L. Warriner, unpublished work), but the maximum is very flat. It may be argued that the bistriethylammonium compounds may not be acting in exactly the same way as the bistrimethyl compounds. Ing & Gill (1958) suggest that the bistriethyl compounds do not become attached to the 'anchoring site' in ganglia.

The occurrence of maxima in activity at different points in different homologous series of these double-ended molecules is difficult to fit in with the idea that the most active compounds combine with two

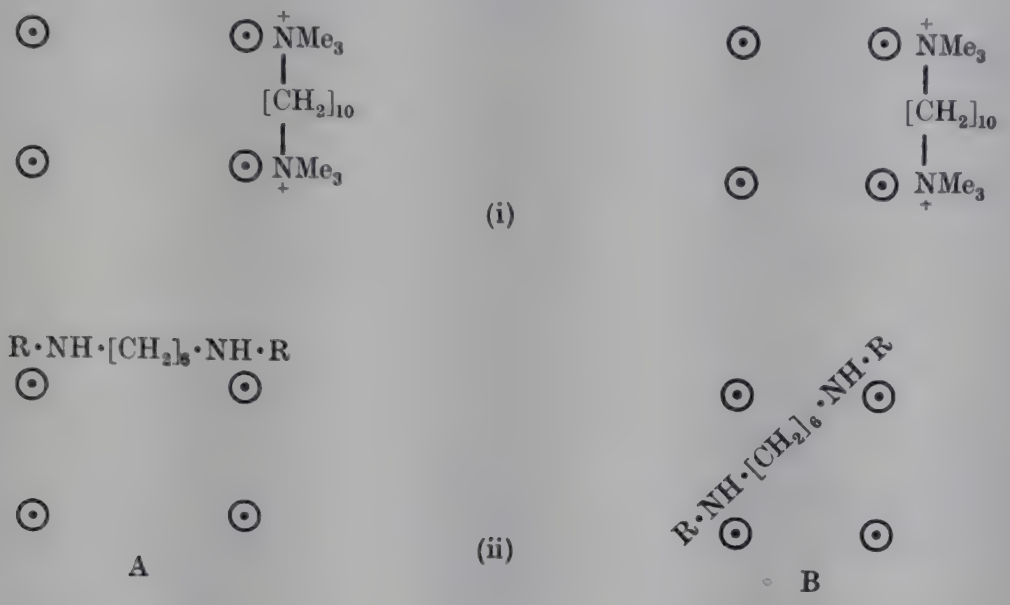


Fig. 5. (i) Possible attachment of decamethonium or suxamethonium ions to receptor units (shown as ⊙). (ii) Possible attachment of polymethylenebiscarbamyl esters of choline; R = $-CO\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}Me_3$.

receptors at once. At the same time it is convenient to explain a sharp maximum in activity by supposing that a particular chain length permits attachment of both ends of the molecule to the biological surface. The explanation may be that the compounds do not combine with two receptors but, as suggested by Ing & Gill (1958) in ganglia, with one receptor unit and an anchoring site, which has different properties from those of the receptor unit. It is also possible that there may be a number of anchoring sites of different types at which these long molecules may become attached.

This idea is, on biochemical grounds, much more acceptable than the idea of attachment at two receptor units. Enzyme studies (see, for example, Dixon & Webb, 1958) indicate that the number of active centres per molecule of enzyme protein is very low, often of the order of one; although the situation in an area so specialized as the end-plate region of a muscle cell may be different, it seems very unlikely that they

are grouped so closely together as depicted in Fig. 5. On the other hand, there could very well be a number of groups on the biological (presumably protein) surface which might provide a second point of attachment of the long molecules.

Because of the complicated nature of pharmacological tests it might be much more suitable to study the problem on an enzyme system such as a cholinesterase. Many of the compounds discussed above are substrates or inhibitors (sometimes both) of cholinesterases; these properties often complicate the interpretation of the pharmacological results.

Table 7. *Hydrolysis of polymethylenebis(acetoxyethyldimethylammonium) salts (mM), by acetylcholinesterase of dog's caudate nucleus (Barlow, 1955b)*

$$CH_3 \cdot CO \cdot O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}Me_2 \cdot [CH_2]_n \cdot \overset{+}{N}Me_2 \cdot CH_2 \cdot CH_2 \cdot O \cdot CO \cdot CH_3$$

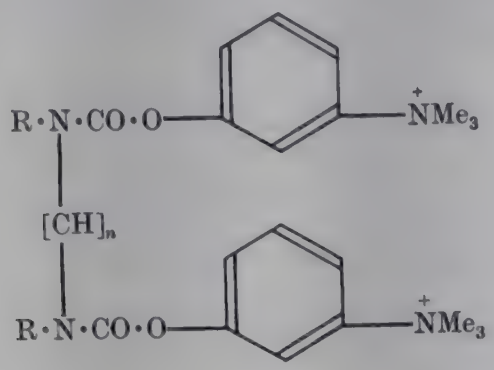
Temperature, 37°.

n	μl. of CO_2/6 min.	n	μl. of CO_2/6 min.
4	0	9	36
5	4	10	29
6	8	11	12
7	30	12	9
8	61		

The cholinesterases have been reviewed by Whittaker (1951) and by Nachmansohn & Wilson (1951)—see also Webb in this volume (p. 90). In acetylcholinesterase there are two receptor groups in the receptor unit, an 'anionic site' and an 'esteratic site'. Recent work, with substrates or inhibitors which have a rigid 5- or 6-membered ring structure, has largely confirmed the ideas of Nachmansohn & Wilson about the nature of the receptor groups and their distribution in the receptor unit (Friess & McCarville, 1954*a*, *b*; Baldridge, McCarville & Friess, 1955; Friess & Baldridge, 1956*a*, *b*, *c*; Friess, Patchett & Witkop, 1957; Friess, 1957; Masterson, Friess & Witkop, 1958; Holland, Durant, Friess & Witkop, 1958).

Polymethylenebis(acetoxyethyldimethylammonium) salts (XIII) were hydrolysed by the acetocholinesterase of dog's caudate nucleus, but they also reduced the rate of hydrolysis of acetylcholine itself in mixtures of the two (Barlow, 1955*b*). They can be regarded as being a biochemical example of partial agonists as described by Stephenson (1956). Table 7 shows the rates of hydrolysis; the octamethylene compound was the most rapidly destroyed. There was not, however, a great increase in the rate of hydrolysis at this chain length, comparable with the great increase in acetylcholine-like activity at the decamethylene compound, and the inhibition of the hydrolysis of acetylcholine continued to rise in the higher members of the series.

The anticholinesterase activity of polymethylenebisquinolinium salts was found to rise steadily from the hexamethylene to the decamethylene compound (Barlow & Himms, 1955), and the anticholinesterase activity of the biscarbamylcholine derivatives (XIV) is maximal in the octamethylene compound (Table 8; Klupp *et al.* 1953). Very high activity is also shown by the octamethylenebiscarbamyl ester of *m*-hydroxyphenyltrimethylammonium (XV) and by the decamethylenebismethyl-



(XV: R = H; $n = 8$)
(XVI: R = Me; $n = 10$)

carbamyl ester (XVI: Kraupp, Schwarzacher & Stumpf, 1955). These results do not yield any satisfactory information about the nature and position of possible anchoring sites on the cholinesterase surface. The problem requires further and more detailed study, with particular attention, for example, to enzyme kinetics.

Table 8. *Inhibition of acetylcholinesterase of dog's caudate nucleus by polymethylenebiscarbamyl esters of choline*

$$Me_3\overset{+}{N}\cdot CH_2\cdot CH_2\cdot O\cdot CO\cdot NH\cdot [CH_2]_n\cdot NH\cdot CO\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}Me_3$$

Substrate, acetylcholine; temperature, 37°; pI_{50}, logarithm of concentration producing 50% inhibition (Klupp, Kraupp, Stormann & Stumpf, 1953).

n	pI_{50}	n	pI_{50}
2	3·8	10	5·7
4	4·2	Eserine	6·6
6	4·9	Carbachol	2·5
8	6·5		

Compounds which imitate or antagonize the actions of acetylcholine are in some ways a bad choice for illustrating how steric factors may influence pharmacological activity. There is no indication as to what enzyme the acetylcholine receptors may be located on, and there is even the possibility that the mechanism by which acetylcholine alters the ionic permeability of the end-plate region of the cell is non-enzymic, that acetylcholine may bring about some change in protein configuration which is followed by enzymic reactions rather than is a consequence

of them. The pharmacological tests involved in the actions of acetylcholine, however, are relatively simple and direct. Many properties can be studied on isolated preparations. Consequently the influence of steric factors on acetylcholine-like activity (or antagonism of acetylcholine) can be discussed with more confidence than can their influence on activity on, say, some structure in the central nervous system. Nevertheless, even simple isolated preparations are much more complicated than isolated enzyme systems, and the pharmacologist has much to learn from the biochemist about steric aspects of drug action.

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CIS–TRANS ISOMERS OF RETINENE IN VISUAL PROCESSES

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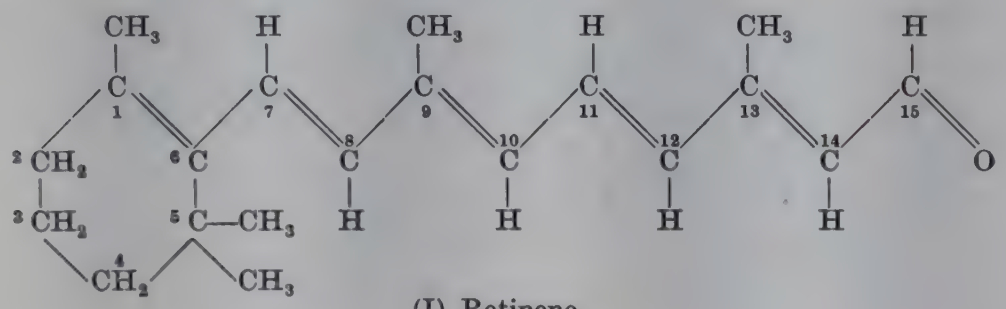
The visual pigments are the photoreceptor molecules of eyes. All those of which we have chemical knowledge consist of a retinene (vitamin A aldehyde) joined to a protein. These protein moieties, though not identical, have many points in common, and have been given the general name of opsins. The retinene part of the molecule is necessary for the chromophoric group, i.e. the part of the molecule which absorbs the light.

Visual pigments fall into several groups. On chemical grounds they are divided into those based on retinene$_1$ (I), the aldehyde of vitamin A_1, and those based on retinene$_2$, derived from vitamin A_2, which has an extra double bond between C-3 and C-4 in the ring. Visual pigments of



(I) Retinene

vertebrates can be further divided into those found in the rods of the retina, concerned with scotopic (low-intensity) vision, and those found in the cones, involved in photopic (daylight) vision. Many visual pigments have been described (see Crescitelli, 1958; Morton & Pitt, 1958), but chemical investigations have concentrated on rhodopsin, the retinene$_1$ pigment which occurs in vertebrate rods and also in some invertebrates. (The terms retinene and vitamin A are used below to mean retinene$_1$ and vitamin A_1.)

Theoretically, 16 stereoisomers of retinene (or vitamin A) are possible, as the isoprenoid side chain has four double bonds, but from the predictions of Pauling (1939, 1949) it appeared that the majority could not exist. According to Pauling, the double bonds in the 7- and 11-positions would not be expected to assume readily the *cis* configuration owing to steric hindrance from the methyl branch groups, which would twist the molecule and so inhibit resonance. If this were so, only the 9- and 13-double bonds could exist in stable *cis* forms and so there

would be only four isomers: all-*trans* (I); 9-mono*cis* (III); 13-mono*cis*; and 9:13-di*cis*. These four isomers of retinene and vitamin A have been synthesized (Robeson, Blum, Dieterle, Cawley & Baxter, 1955; Robeson, Cawley, Weisler, Stern, Eddinger & Chechak, 1955), but, besides these four 'allowed' isomers, it has been possible to prepare two more retinenes (and vitamins A) with a *cis* link in the 'hindered' 11-position. These are 11-mono*cis* and 11:13-di*cis* (Oroshnik, 1956). Clearly the 11-*cis* forms are not as hindered as was expected, although molecular models confirm that both the 11-*cis* and 11:13-di*cis* are twisted slightly owing to the predicted steric hindrance. Models can be made of two more 11-*cis* forms which have not yet been synthesized, namely, 9:11-di*cis* and 9:11:13-tri*cis*; the former is markedly twisted, and the latter very severely distorted.

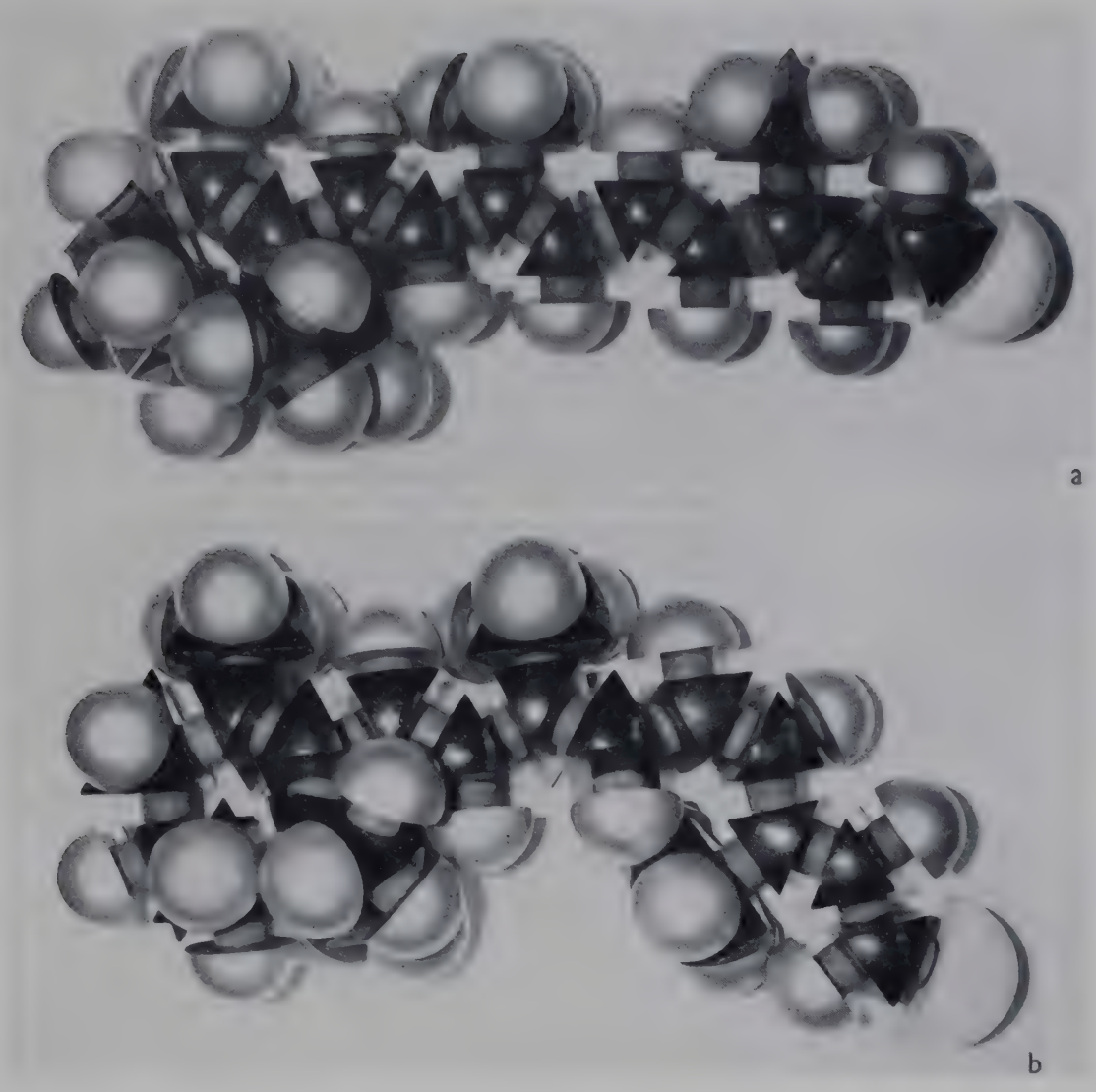
Wald, Brown, Hubbard & Oroshnik (1955) tried to synthesize 7-*cis*-vitamin A, but failed to do so, and indeed it is quite impossible to build molecular models of 7-*cis* isomers owing to interference by the methyl group on position 9 with those of the ring (see the models shown in Plate 1).

Although Pauling's predictions about the number of *cis–trans* isomers have proved to be unduly restrictive, they are right in the sense that the total number of known isomers falls well short of the theoretical limit of 16. Six forms have been prepared; from models it appears that of the remaining possibilities only the 9:11-di*cis* isomer could perhaps be formed, and even so its stability would be doubtful.

Of the isomeric retinenes which can be synthesized, it is not known how many occur in Nature—retinene is rarely found in the free state. Vitamin A, on the other hand, occurs widely and in various isomeric forms. The thermodynamically stable isomer is the predominating all-*trans*; 13-*cis* is a constituent of many fish-liver oils, although Lambertsen & Braekkan (1956) have produced evidence that it is formed from all-*trans*-vitamin A during storage and processing; 9:13-di*cis* has been reported in the eyes of the prawn *Pandalus bonnieri* (Barnholdt & Hjarde, 1957); and it appears that 9-*cis* occurs in the liver oil and plasma of some animals (Wald, 1957), perhaps in part as an artifact (Hubbard & Wald, 1953; Hubbard, 1956*a*). Of the 'hindered' isomers, 11-*cis*-vitamin A is found in the eyes of cattle (Krinsky, 1958) and of some invertebrates, at times with the 11:13-di*cis* isomer as well (reviewed by Fisher & Kon, 1959). Although it is not known whether they occur naturally, the corresponding retinenes have been prepared from all these forms of vitamin A, and they are reasonably stable, except perhaps the 11:13-di*cis* isomer (Wald, Brown, Hubbard & Oroshnik, 1955; Oroshnik, Brown, Hubbard & Wald, 1956).

The isomeric retinenes are readily interconvertible in solution by the

PLATE I



Molecular models of **a**, all-*trans*-retinene; **b**, 11-*cis*-retinene.

action of light. Iodine may be used as a catalyst (Hubbard, 1956*b*), but is not necessary; light by itself is very effective (Hubbard, Gregerman & Wald, 1953). All the isomeric retinenes are converted by light into apparently the same equilibrium mixture of isomers. It is hard to be quite certain of this, since light tends to destroy retinene to some extent (see Hubbard, 1956*a*) and prolonged irradiation may be necessary as the ethylenic linkages isomerize at varying rates. The results of Hubbard *et al.* (1953) and Hubbard (1956*b*) show that the 11- and 13-linkages are rapidly isomerized by light, whereas the 9-link changes only slowly. The products formed depend to some extent on the time of illumination.

Some retinenes can also be isomerized thermally, e.g. by heating at 70° for 3 hr. (Hubbard *et al.* 1953; Wald, Brown, Hubbard & Oroshnik, 1955). The main thermal effect seems to be a change from 11-*cis* to -*trans* (see also Oroshnik *et al.* 1956); the 9-*cis* link is hardly affected at this temperature (Wald, Brown, Hubbard & Oroshnik, 1955). There is also evidence that in some conditions the 13-*cis* link can readily isomerize in the dark (Ames, Swanson & Harris, 1955). At physiological temperatures, therefore, thermal reactions may modify isomerizations of retinene initiated by light.

The broad picture is nevertheless fairly clear. The action of light on all-*trans*-retinene will produce some 11-*cis* with a smaller proportion of 9-*cis*, and the amount of the latter will depend on the time of exposure to light (Hubbard *et al.* 1953; Hubbard, 1956*a*); some 13-*cis* will also be formed (see Brown & Wald, 1956). 11-*cis*-Retinene is photoisomerized to give mainly all-*trans*; 13-*cis* gives all-*trans* which undergoes subsequent isomerization; 9:13-di*cis* rapidly gives 9-mono*cis*, which isomerizes only very slowly.

These findings provide a guide when predicting the action of light on retinene isomers in combination with opsins, but it is difficult to deduce quantitative relationships applicable to pigments in the eye itself. The solvents used *in vitro* affect the proportions of isomers formed (Brown & Wald, 1956), and when retinene is in combined form, e.g. as oxime (Hubbard, 1956*b*), or attached to opsin (Hubbard & Kropf, 1959), its isomerization differs from that observed with free retinene.

In the presence of denatured opsin (and probably other substances), 11-*cis*- and 9-*cis*-retinene are isomerized thermally much more rapidly than when alone in solution (Hubbard, 1959). Nevertheless, these isomerization studies provide the best available data from which to predict the action of light on retinene isomers combined with opsins in visual pigments.

Most of the aforementioned conclusions were based on spectroscopic evidence. The various isomers of retinene can be distinguished to some extent by their absorption spectra (see Hubbard *et al.* 1953; Hubbard,

1956*a*; Wald, Brown, Hubbard & Oroshnik, 1955; Robeson, Blum, Dieterle, Cawley & Baxter, 1955; Brown & Wald, 1956); briefly, *cis* isomers have lower extinction coefficients, and the absorption maximum is at a shorter wavelength than that of the all-*trans* isomer (see, for example, Fig. 1). On isomerization all-*trans*-retinene shows a fall in intensity of absorption with a shift of its maximum to shorter wavelengths; *cis* isomers usually display opposite effects (see Fig. 1) in non-polar solutions. Spectroscopic work of this type can be done on very

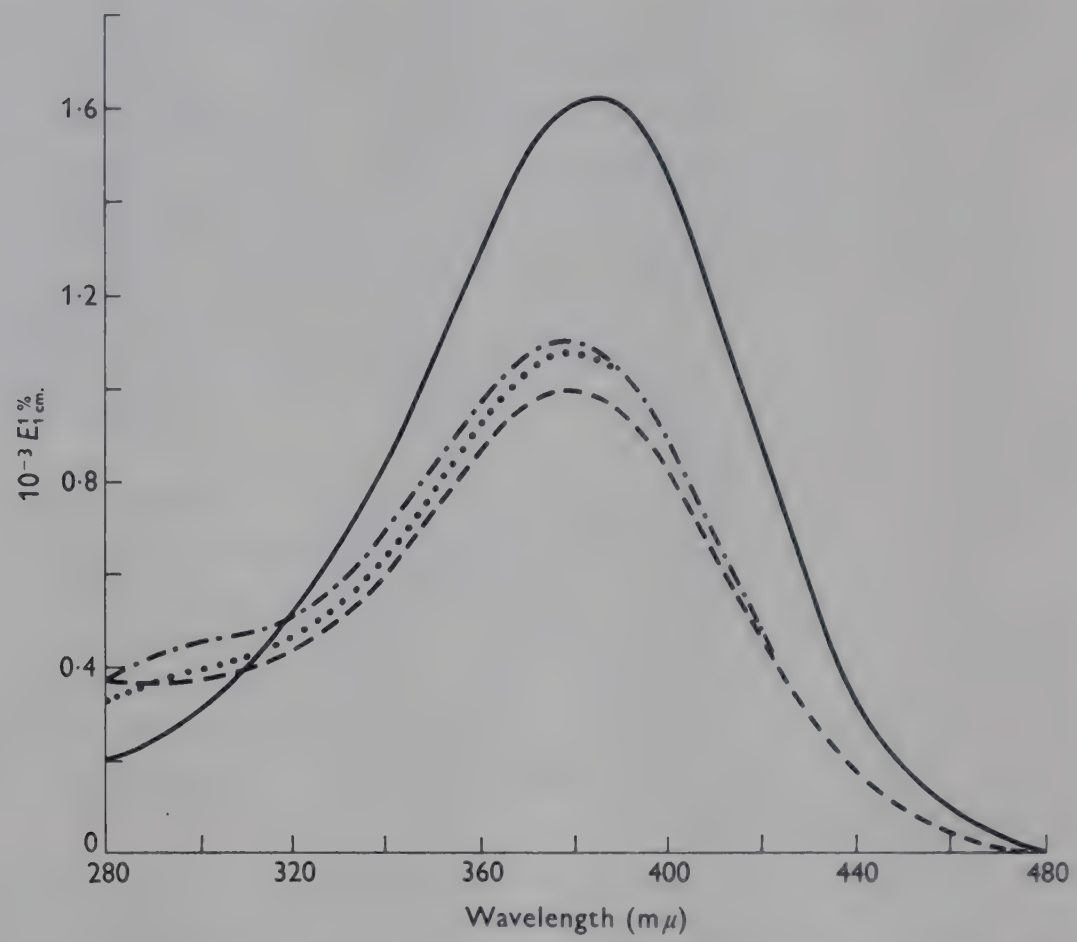


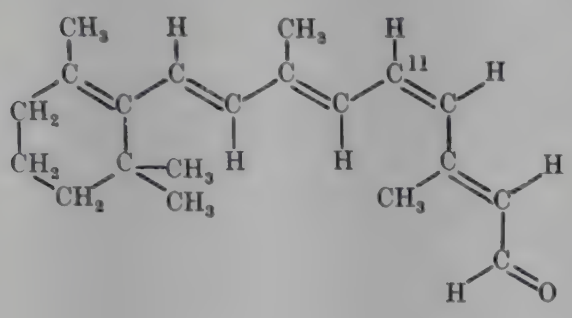
Fig. 1. Effect of exposure to light on the spectra of all-*trans*-retinene (——, before; – · – · – · –, after) and 11-*cis*-retinene (– – –, before; . . . . after). (Equilibrium not fully attained.) In ethanolic solution (Hubbard, Gregerman & Wald, 1953).

dilute solutions (of the order of 10^{-5} M). This has proved very valuable in the study of visual pigment preparations, which usually contain very small amounts of material.

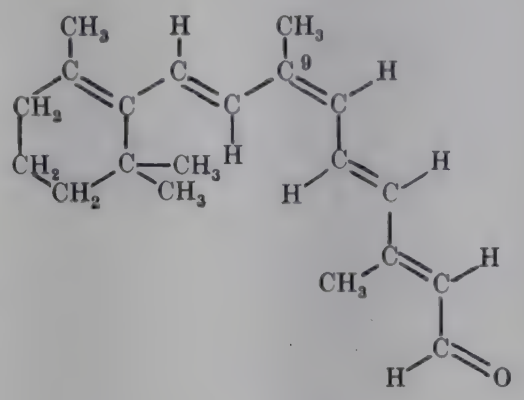
The importance of *cis–trans* isomerism in vision was first realized when Hubbard & Wald (1953) found that the origin of the vitamin A used for making retinene affected the efficacy *in vitro* of the product. Thus they observed that retinene made from synthetic all-*trans*-vitamin A did not join with the protein opsin to give the visual pigment rhodopsin; but if this retinene was exposed to the isomerizing action of light, a part of it could form rhodopsin. A long and complicated series of investigations

(reviewed by Morton & Pitt, 1957) was necessary to solve the major problems raised by this work. It was eventually established that only one isomer of retinene would unite with opsin to give rhodopsin. It was the 11-*cis* form (II). The only other isomer which would react with opsin was the 9-*cis* form; this gave a compound called by Hubbard & Wald (1953) isorhodopsin. Although it closely simulates visual pigments this isorhodopsin has not been found occurring naturally in eyes.

Although, as described above, a number of isomers of retinene can exist, and might conceivably be found in eyes, most discussions on isomeric retinenes in the chromoproteins in the eye have been restricted to the three isomers which have been demonstrated to be involved in some way: all-*trans* (I); 11-*cis* (II); and 9-*cis* (III). This limitation may



(II) 11-*cis*-Retinene



(III) 9-*cis*-Retinene

prove to be slightly too restrictive, but it is not basically inadequate. In fact it provides a satisfactory framework for interpreting the data.

The introduction of a *cis* linkage at the 11-position changes markedly the shape of the retinene molecule (Plate I). The all-*trans* isomer has a straight side chain; the 11-*cis* form is bent. The 9-*cis* shows a similar bending to that of the 11-*cis*, but the kink has moved along the chain. Presumably it is this shape which is necessary for union with opsin to form a pigment (depicted schematically in Fig. 2). The straight chain of the all-*trans* isomer prevents this, as does bending at the 13-position (for molecular models of some of these isomers, see Hubbard & Wald, 1953).

Rhodopsin has an absorption maximum in the visible region near 500 mμ (Fig. 3), although the exact position varies from species to species. When rhodopsin (in solution) is illuminated, it breaks down to retinene and the protein opsin. As the absorption peak of retinene is in the ultraviolet region, the action of light results in the disappearance of the red–purple colour of rhodopsin to give an almost colourless solution (Fig. 3); the process is usually referred to as the bleaching of rhodopsin. For many years it has been presumed that this is what goes

on in the eye when light strikes rhodopsin. Rhodopsin must be regenerated *in vivo* at a reasonably fast rate, or all the pigment would be bleached away, and so vision would stop. The regeneration can be brought about *in vitro* by mixing opsin and retinene in the dark.

When Hubbard & Wald (1953) investigated the regeneration and bleaching of rhodopsin, they found that the two processes were not simple reversals. 11-*cis*-Retinene was required to join with opsin to form rhodopsin, but the bleached rhodopsin solutions contained opsin

Fig. 2. Postulated fit of opsin with isomeric retinenes (schematic): ——, 11-*cis*; . . ., 9-*cis*; – – –, all-*trans*. All retinenes are shown attached to the opsin by their aldehyde group at the right-hand end of the molecule (Hubbard, 1958*a*).

and all-*trans*-retinene. An excellent demonstration of this was given by Wald & Brown (1956). A solution of cattle opsin was mixed with 11-*cis*-retinene and the spectrum of the mixture recorded at various times (see Fig. 4 *A*). The ultraviolet peak of the retinene disappeared, and the characteristic absorption maximum of rhodopsin rose in the visible region of the spectrum. When regeneration of rhodopsin was complete, the same solution was exposed to orange light for short periods of time (see Fig. 4 *B*). The rhodopsin peak disappeared and, as bleaching went on, the ultraviolet absorption of retinene became more prominent. The final solution, containing only retinene and opsin, had a much higher ultraviolet peak than had the original solution of retinene and opsin. Initially in the 11-*cis* form, the retinene in the final solution was largely

all-*trans*, which absorbs more intensely. The processes of formation and bleaching of rhodopsin had resulted in an isomerization of the retinene.

Rhodopsin is formed from 11-*cis*-retinene, but, as mentioned above, the 9-*cis* isomer will also unite with opsin to give isorhodopsin. This has its absorption peak displaced to shorter wavelengths from that of rhodopsin; cattle rhodopsin shows $\lambda_{max.}$ at 498 mμ, cattle isorhodopsin at 487 mμ (Hubbard & Wald, 1953). Isorhodopsin has never been isolated from eyes and has been found only in solutions of visual pigments which have been exposed to light in rather unusual ways (see below), or when artificially synthesized *in vitro* from opsin and 9-*cis*-retinene; it

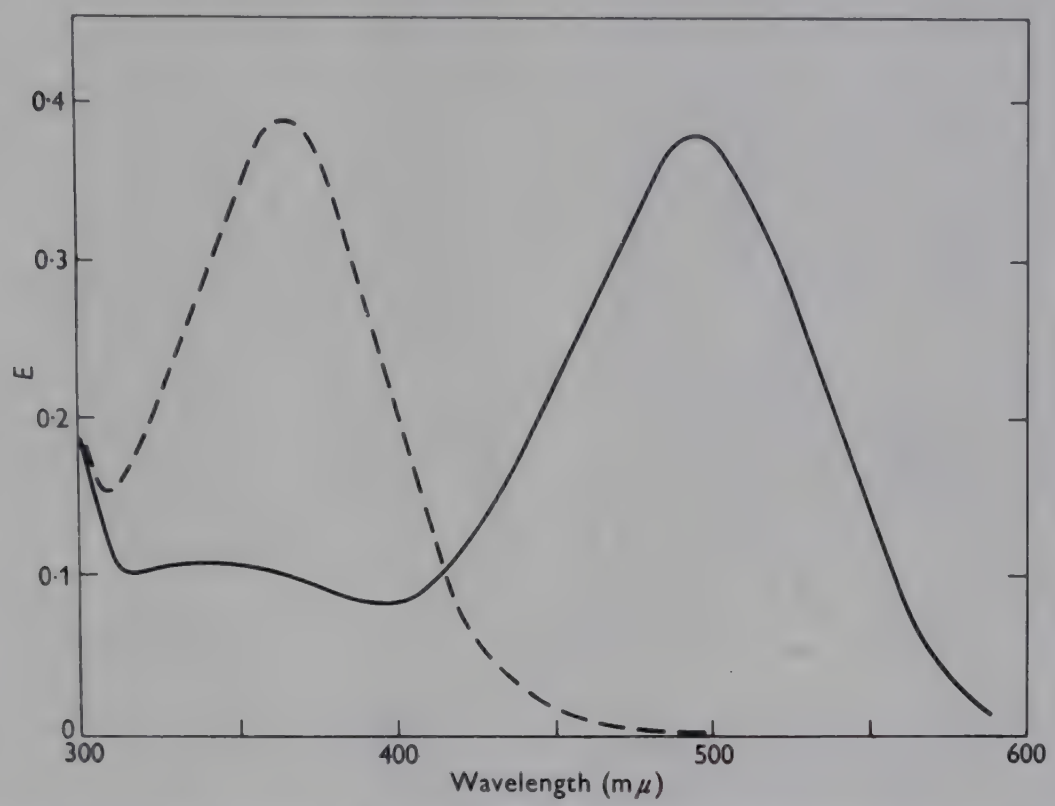


Fig. 3. Absorption spectrum of cattle rhodopsin in digitonin solution (——). Absorption spectrum after exposure to light, pH 9·2 (– – –) (from Collins, Love & Morton, 1952).

seems not to be a normal visual pigment in the species which have been studied (Wald, 1957). Isorhodopsin undergoes an isomerization analogous to that of rhodopsin; when bleached it yields opsin and all-*trans*-retinene. Even though it be an artifact and of no physiological importance, isorhodopsin when found in visual pigment preparations often provides valuable experimental evidence of isomerization (e.g. Adams, Kennedy, Wulff & Zonana, 1958).

The all-*trans*-retinene liberated by the bleaching of rhodopsin does not re-form rhodopsin when mixed with opsin *in vitro*. Yet there is evidence that, in rather more complex systems, the all-*trans* isomer can serve to some extent for rhodopsin regeneration (Bliss, 1951; Hubbard & Wald, 1951; Collins, Green & Morton, 1953, 1954) as indeed it must *in vivo*.

Hubbard (1956*a*) clarified this when she found in the retina an enzyme, retinene isomerase, which will change all-*trans*- to 11-*cis*-retinene. In the dark, retinene isomerase converts all-*trans*- or 11-*cis*-retinene to a mixture of about 5% 11-*cis* and 95% all-*trans*. In the light, the rate of formation of all-*trans* is increased several times and a new steady-state is attained in which 11-*cis* constitutes about a third of the mixture.

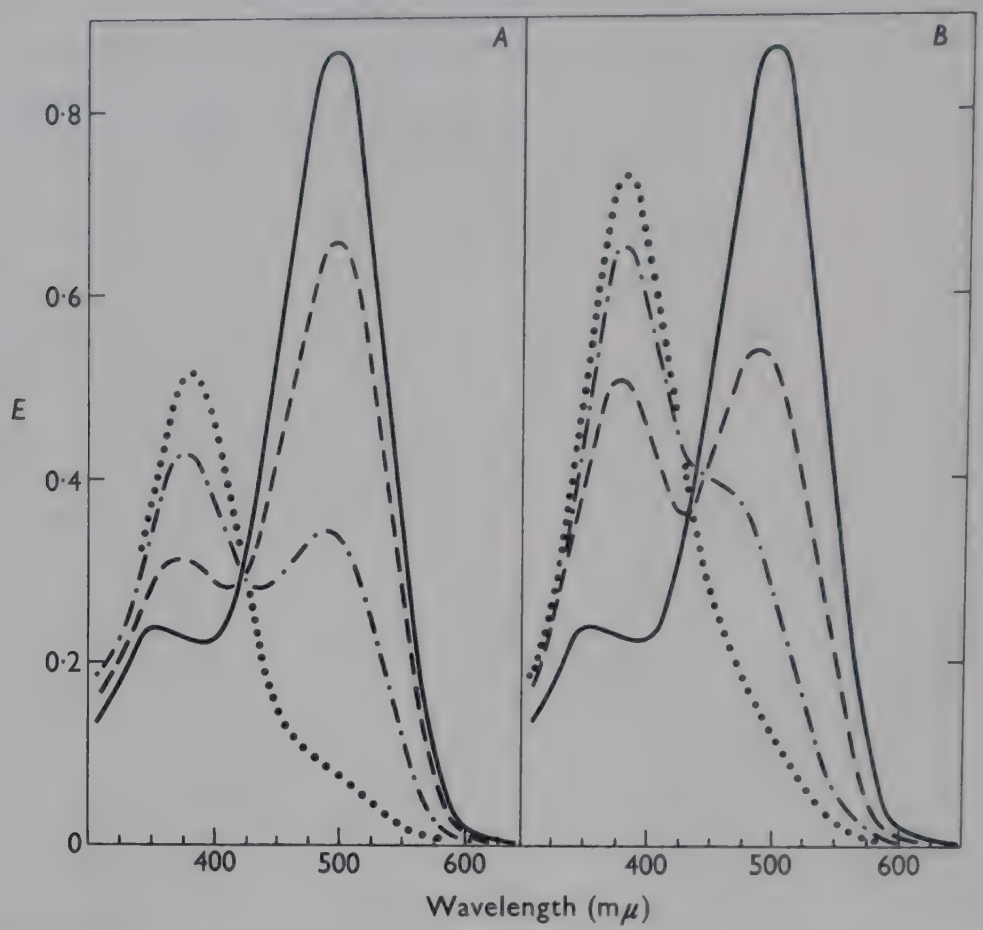


Fig. 4. *A*, Synthesis of rhodopsin in solution. Cattle opsin and 11-*cis*-retinene incubated in the dark. Absorption spectra determined after 18 sec. (. . .); after 5 min. (– · – · –); after 30 min. (– – –); after 3 hr. (——). *B*, The rhodopsin formed (absorption spectrum, ——) is bleached by exposure to light of wavelength > 550 mμ for various periods. Absorption spectra determined after irradiating for a total of 10 sec. (– – –); 30 sec. (– · – · –); and 120 sec. (+45 sec. by light of wavelength > 440 mμ) (. . .) (Wald & Brown, 1956).

Light influences both the rate of the enzymic reaction and the nature of the products; presumably the thermodynamic equilibrium is that brought about in the dark, and light supplies energy to give a different steady-state mixture. The effect of light is not just a simple photo-isomerization on top of the 'dark' enzymic reaction, for the combination of isomerase and light works more rapidly and gives a more favourable yield of 11-*cis*-retinene than the sum of the two separate reactions. Interconversions catalysed by the isomerase, with or without light, are distinguished from simple photoisomerizations by the specificity of the changes. Retinene isomerase will act only on all-*trans*- and 11-*cis*-retinene. It will neither act on nor produce 9-*cis*- or 13-*cis*-retinene.

The exact physiological status of retinene isomerase has not yet been firmly established, for without light it does not *in vitro* form 11-*cis*-from all-*trans*-retinene fast enough to account for the rate of regeneration of rhodopsin observed in the living eye (Hubbard, 1956*a*). With light this difficulty vanishes, but it seems improbable that enough light of the wavelengths absorbed by retinene is transmitted by the lens of the eye in many species. Lewis (1957) has deduced that in the rat the rate of regeneration of rhodopsin is the same in the light as in the dark. The work of Rushton (1958) also implies that light cannot greatly affect the action of retinene isomerase in normal visual processes. He bleached the rhodopsin in a human subject's eye with blue light and with yellow light, and measured the rate of regeneration of rhodopsin in the eye by his ophthalmoscopic method. The blue light would be absorbed by retinene ten times more effectively than the yellow light, and therefore would be potentially much more effective in activating the isomerase. There was no difference in rhodopsin-regeneration rates after the two bleachings, indicating that photoactivation of retinene isomerase is not operating under physiological conditions.

This conclusion would not be valid if the eye contained large stores of the 11-*cis* isomer. Free retinene does not appear in the eye in large amounts (Krinsky, 1958), but many animals have reserves of vitamin A in the eye. There are species differences: among mammals, cattle and rabbits have eye reserves of the vitamin; dark-adapted rats do not. In cattle, the reserves (in molar terms) amount to less than the rhodopsin content (Peterson, 1957; Pitt, unpublished work). Krinsky (1958) has reported that the greater part of this is in the 11-*cis* form; estimates from this Laboratory (Peterson, 1957) are somewhat lower, but indicate that about a third is in the 'active' configuration.

Rushton (1958), however, has shown that in his experimental subjects the demand on the 11-*cis*-retinene was such that any eye reserves would rapidly have been exhausted in the absence of fairly rapid reisomerization of the all-*trans*-retinene liberated by bleaching.

The most plausible assumption is that, in the eye, retinene isomerase is working without photoactivation and that *in vivo* it works more rapidly than in Hubbard's (1956*a*) simplified *in vitro* system. The visual isomerization cycle can therefore be represented in outline by Fig. 5.

The most recent investigations on this cycle have aimed at finding where and how retinene is isomerized from 11-*cis* to all-*trans* during the processes of rhodopsin formation and bleaching.

It has not been possible to detect any intermediates in the formation of rhodopsin from 11-*cis*-retinene and opsin, but its bleaching in solution involves a number of stages before free retinene splits off the protein

opsin (Fig. 6). These intermediates can be shown to accumulate by changing the temperature or the pH at which bleaching is done.

Wald, Durell & St George (1950) found that illumination of rhodopsin at low temperatures (below $-40°$) changed the spectrum only slightly,

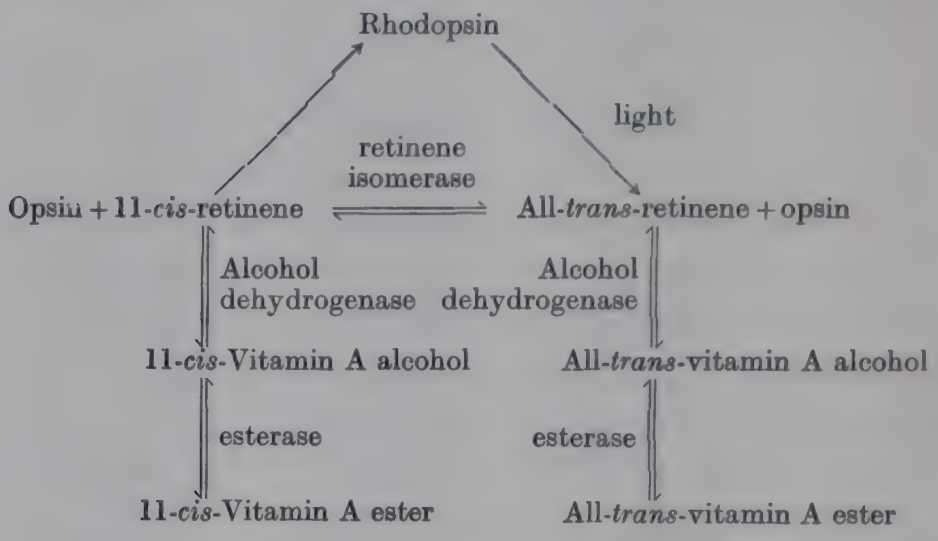


Fig. 5. Isomeric forms of retinene and vitamin A in the eye.

giving a product which they called lumirhodopsin. This was the only photochemical step in the bleaching of rhodopsin; the subsequent breakdown was thermal. Warming the solution from $-40°$ to $-20°$

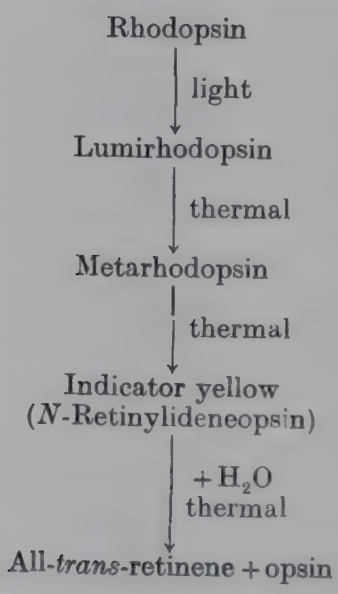


Fig. 6. Intermediates in the breakdown of rhodopsin.

allowed the formation of another product, called metarhodopsin, which differed spectroscopically only slightly from lumirhodopsin. The solution was still coloured; no bleaching had occurred. Metarhodopsin hydrolyses in solution at normal working temperatures and rapidly breaks down via indicator yellow (*N*-retinylideneopsin) to retinene and opsin; this reaction is the true bleaching process.

Rhodopsin occurs not only in vertebrates, but also in invertebrates such as the squid (St George & Wald, 1949; Hubbard & St George, 1958), octopus, cuttlefish (Brown & Brown, 1958) and lobster (Wald & Hubbard, 1957). The study of the action of light on rhodopsin was much facilitated when Hubbard & St George (1958) confirmed earlier reports that squid rhodopsin did not normally bleach in light. They investigated the phenomenon in detail; light affected squid rhodopsin but did not bleach it except in alkaline solution. In physiological conditions, the breakdown of squid rhodopsin by light stopped at an intermediate stage, apparently at the level of metarhodopsin.

Most of the work on invertebrate rhodopsins has been done on that of the squid (Hubbard & St George, 1958), but other invertebrate rhodopsins seem similar (Kropf, Brown & Hubbard, 1959). Squid rhodopsin on illumination with light at $-65°$ gives lumirhodopsin; when allowed to warm up to room temperature, it forms metarhodopsin. This, in contrast with vertebrate metarhodopsins, is stable and is also a pH indicator. Indicator behaviour had previously been noted only with indicator yellow (*N*-retinylideneopsin), which is a Schiff's base, capable of forming a conjugate acid at low pH values (Pitt, Collins, Morton & Stok, 1955). Presumably squid metarhodopsin resembles in some ways a Schiff's base. It appears to have a pK_a of about 7·7; on the acid side of this it has an absorption maximum at 500 mμ. This form is referred to as acid metarhodopsin, even though it is the one which predominates in neutral solutions. On the alkaline side, the maximum absorption is at 380 mμ (alkaline metarhodopsin) (Fig. 7).

When squid rhodopsin is changed to metarhodopsin in neutral solution, no bleaching occurs. In alkali it can be bleached by illumination with monochromatic light of about 500 mμ, thus converting it all into alkaline metarhodopsin. The bleaching completed, the pH can be lowered to change the original rhodopsin quantitatively to acid metarhodopsin. This process is depicted in Fig. 7. The maximum of acid metarhodopsin is near that of squid rhodopsin, but at slightly longer wavelengths (500 and 493 mμ); the molecular extinction coefficient of acid metarhodopsin (ϵ 60 000) is, however, much higher than that of squid rhodopsin (ϵ 40 600). These spectroscopic relationships are very similar to those seen between all-*trans*- and 11-*cis*-retinene (see Fig. 1) (Hubbard & St George, 1958).

Hubbard & Kropf (1958) proved clearly that the retinene portion of the molecule had been converted from 11-*cis* in rhodopsin into all-*trans* in metarhodospin by heat instead of light. Heat denatured the opsin part of the molecule, releasing the retinene with very little isomerization (Hubbard, 1958*b*, 1959). The retinene set free from rhodopsin was mainly in the 11-*cis* configuration, and that from metarhodopsin

mainly in the all-*trans* form (Hubbard & Kropf, 1958; Kropf & Hubbard, 1958). In practice, a proportion of other isomers was formed, as the heating necessary to denature the protein, although very mild, resulted in some isomerization of the liberated retinene, but not to an extent to confuse the results (Hubbard, 1959). The experiment showed directly that isomerization had taken place in going from rhodopsin to metarhodopsin. [The apparently excellent agreement of the spectra (Fig. 7) with what would be expected in theory from a *cis* to *trans* change (cf. Fig. 1) seems from the later work of Kropf *et al.* (1959) to be largely fortuitous.]

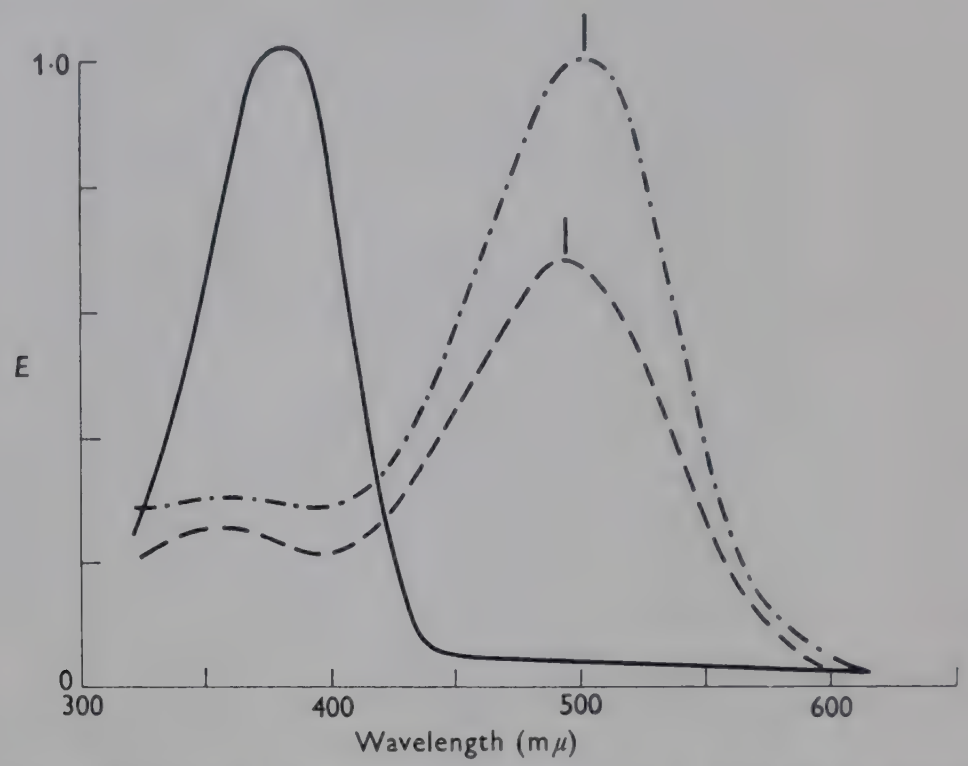


Fig. 7. Absorption spectra of (*a*) squid rhodopsin (– – –) ($\lambda_{max.}$ 493 mμ); (*b*) rhodopsin after irradiation with orange light at pH 9·9 (alkaline metarhodopsin, $\lambda_{max.}$ 380 mμ) (——); (*c*) of bleached alkaline solution brought to pH 5·5 (acid metarhodopsin, $\lambda_{max.}$ 500 mμ) (– · – · –). At 5° (Hubbard & St George, 1958.)

The action of orange light on squid rhodopsin in alkaline solution, followed by acidification, results in a quantitative conversion of rhodopsin into alkaline metarhodopsin. If, however, acid metarhodopsin is irradiated and then made alkaline, rather less than 60 % of the expected alkaline metarhodopsin is obtained, and an absorption peak remains in the visible region with $\lambda_{max.}$ 493 mμ (Fig. 8). This is due to rhodopsin; it has been formed from acid metarhodopsin by the action of light. The photoconversion of rhodopsin into metarhodopsin is reversible. If either squid rhodopsin or acid metarhodopsin is irradiated in slightly acid solution, the final mixture is always the same: 42–47 % rhodopsin; 58–53 % acid metarhodopsin (Fig. 8). Starting with either, light produces the identical pseudo-equilibrium of isomers. Rhodopsin therefore

cannot be converted quantitatively into acid metarhodopsin by simple illumination. It is possible to demonstrate such a quantitative conversion only via alkaline metarhodopsin, by irradiating squid rhodopsin in alkaline solution with orange light which is not absorbed by alkaline metarhodopsin, and so cannot isomerize it (Hubbard & St George, 1958).

One of the most remarkable features of this reversible photoconversion of rhodopsin and acid metarhodopsin is its stereospecificity. In the steady-state mixture, almost half the chromophoric groups are in the 11-*cis* configuration (i.e. as rhodopsin); the remainder are predominantly in the all-*trans* form (Hubbard & St George, 1958; Hubbard

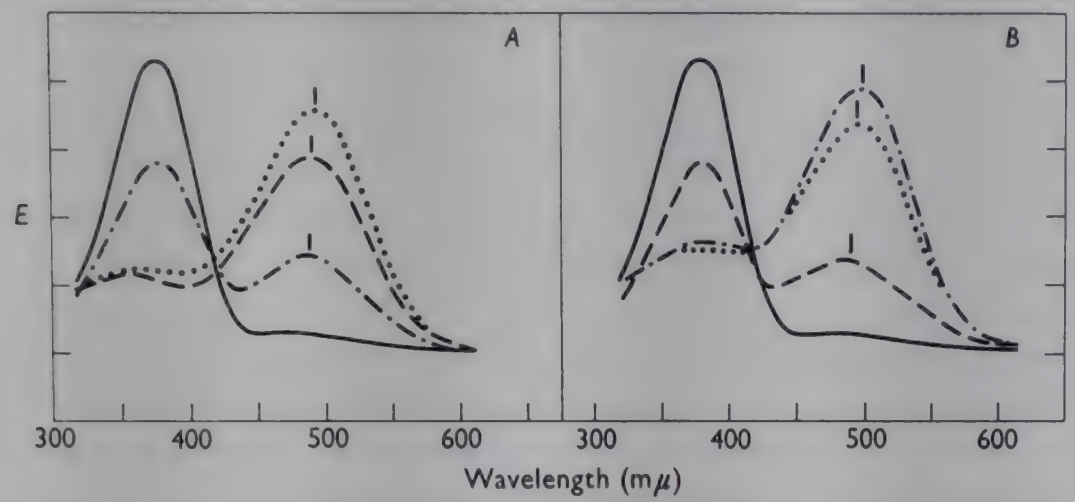


Fig. 8. Irradiation of squid rhodopsin (*A*) and acid metarhodopsin (*B*) with orange light. Absorption spectra (*A*) rhodopsin (100%) (– – –); after irradiation at pH 6·1 [mixture of rhodopsin (44%) and acid metarhodopsin (56%)] (. . .); irradiated mixture brought to pH 9·5 in the dark [mixture of rhodopsin (44%) and alkaline metarhodopsin (56%)] (– · – · –); after irradiation at pH 9·5 (alkaline metarhodopsin, 100%) (——). *B*, Acid metarhodopsin (100%) (– · – · –); after irradiation at pH 6·1 [mixture of acid metarhodopsin (58%) and rhodopsin (42%)] (. . .); irradiated mixture brought to pH 9·5 in the dark [mixture of rhodopsin (42%) and alkaline metarhodopsin (58%)] (– – –); after irradiation at pH 9·5 (alkaline metarhodopsin, 100%) (——) (Hubbard & St George, 1958).

& Kropf, 1958). If, on the other hand, alkaline metarhodopsin is irradiated, the product contains not only rhodopsin but also isorhodopsin, which was not formed from acid metarhodopsin. Some of the all-*trans* chromophoric groups must have been isomerized to the 9-*cis* form. From alkaline metarhodopsin, the stereoisomerization is not so specific (Hubbard & St George, 1958), but, even so, appears more restricted than does that of retinene itself in simple solution.

It is clear from all this work by Hubbard and her co-workers that the conversion of squid rhodopsin into metarhodopsin involves the photoisomerization of the retinene portion of the molecule and that the process is reversible, since light acts on any one isomer to produce the same pseudo-equilibrium mixture. Squid metarhodopsin differs, however,

from the metarhodopsins of vertebrates which have provided most of the experimental material for work on visual pigments, notably in being a pH indicator, but in other properties too (Hubbard, Brown & Kropf, 1959; Kropf *et al.* 1959). To repeat on vertebrate rhodopsins and metarhodopsins the photoisomerization experiments first done on squid pigments was much more difficult. Work on the squid pigment was facilitated by there being an alkaline form of metarhodopsin with an absorption spectrum very different from that of rhodopsin. Vertebrate metarhodopsins have only one form, with an absorption curve similar to that of rhodopsin. Any light which affects rhodopsin will also affect metarhodopsin. Even if vertebrate rhodopsin isomerizes like squid rhodopsin, irradiating it in conditions in which metarhodopsin is stable can give only a steady-state mixture (cf. squid rhodopsin and acid metarhodopsin, Fig. 8).

The same problem arises in investigations designed to penetrate further into the steps between rhodopsin and metarhodopsin. In the squid this clearly involved a reversible photoisomerization of the retinene portion. Yet earlier work had established that the only photochemical step in the bleaching of vertebrate rhodopsin is the reaction rhodopsin → lumirhodopsin; this was also confirmed for invertebrate rhodopsins by Kropf *et al.* (1959). The photoisomerization observed in going from squid rhodopsin to metarhodopsin must primarily have been brought about in the step rhodopsin to lumirhodopsin. To investigate this, either in vertebrates or invertebrates, brings up the same problems as with vertebrate metarhodopsins: no lumirhodopsin shows indicator properties and all lumirhodopsins have absorption curves overlapping with their parent rhodopsins (Hubbard *et al.* 1959; Kropf *et al.* 1959). Irradiation of rhodopsin at low temperatures gives a steady-state mixture of rhodopsin and its initial photo-products. In such conditions it is impossible to convert all the rhodopsin into its first photo-products.

This realized, some earlier observations fell into place. Wald *et al.* (1950) and Collins & Morton (1950) had independently observed that if rhodopsin was irradiated at low temperatures and allowed to warm up in the dark, only about half the rhodopsin was broken down to retinene and opsin; the rest remained as a mixture of rhodopsin and isorhodopsin. Similar results have since been obtained by Wulff, Adams, Linschitz & Abrahamson (1958) and Adams *et al.* (1958) after flash photolysis of solutions of rhodopsin at room temperature. Hagins (1955, 1957) has observed the same phenomenon in the intact eye of the rabbit; a very brief photoflash, no matter how bright, would not bleach more than half of the rhodopsin initially present. If the flash was repeated immediately, it had no further bleaching effect; if repeated

after an interval, it would never bleach more than 50 % of the remaining pigment.

The explanation of these findings would seem to be that a quantum of light will isomerize the 11-*cis*-retinene portion of rhodopsin to the all-*trans* form and this in turn may be changed by further quanta to give a steady-state mixture of isomeric retinenes, all still attached to the opsin. The 11-*cis* component of this mixture is rhodopsin; the 9-*cis*, isorhodopsin; there will certainly be an all-*trans* and possibly other isomeric chromoproteins. Of the isomeric retinenes only the 11-*cis* and 9-*cis* have the shape to combine with vertebrate opsins to form a stable pigment (see Fig. 2), and so all other isomeric chromoproteins hydrolyse to retinene and opsin, except at low temperatures which inhibit thermal reactions.

At normal temperatures, the unstable fraction of the initial photo-products is bleached; prolonged illumination will make the remaining rhodopsin (and any isorhodopsin) change over to restore the pseudo-equilibrium, and so in time all will be bleached by light. If only a short flash is given, there is no time for the decay of the unstable portion of the initial photo-products.

This concept clearly calls for a reappraisal of nomenclature. Wald *et al.* (1950) irradiated rhodopsin at $-45°$, and gave the product the name lumirhodopsin. On the photoisomerization theory, this will be a mixture of chromoproteins including rhodopsin (11-*cis*), isorhodopsin (9-*cis*) and other isomeric forms unstable at low temperature; the original (vertebrate) metarhodopsin of Wald *et al.* (1950) will also have been a mixture. Hubbard & Kropf (1958) therefore redefined lumirhodopsin and (vertebrate) metarhodopsin as the labile fraction of the mixture of stereoisomeric chromoproteins, which hydrolyses to retinene and opsin above $-15°$.

In vertebrates it is impossible to give such an unequivocal demonstration as in the squid that the retinene is isomerized from 11-*cis* to all-*trans* in going from rhodopsin to metarhodopsin. Hubbard & Kropf (1958) were nevertheless able to show that the process involves a photoisomerization. By irradiating cattle rhodopsin at $-20°$ they were able to convert it into a mixture of rhodopsin, isorhodopsin and metarhodopsin. At this stage the absorption spectrum was plotted. On allowing the mixture to warm up in the dark in the presence of hydroxylamine, the metarhodopsin gave retinene oxime and opsin, the rhodopsin and isorhodopsin remained stable. The mixture was cooled and the spectrum redetermined. The difference between the original and final spectra was due to the conversion of metarhodopsin into retinene oxime. Since retinene oxime does not absorb light above 450 mμ, the difference spectrum at longer wavelengths will be the same as the absorption

spectrum of cattle metarhodopsin. By this means Hubbard & Kropf (1958) found cattle metarhodopsin (at $-20°$) to have $\lambda_{max.}$ 478 ± 2 mμ, i.e. at clearly shorter wavelengths than cattle rhodopsin (502 mμ) or isorhodopsin (490 mμ) at the same temperature (absorption spectra shown by Kropf & Hubbard, 1958). This is the opposite of what is found in squid rhodopsin, and is contrary to what would be predicted for a simple *cis–trans* change of this type.

Table 1. *Compositions of pseudo-equilibria produced by irradiating rhodopsin solutions at* $-20°$ *with monochromatic lights of various wavelengths* (*Hubbard & Kropf*, 1958)

	Percentage of compound in mixture after irradiation at		
Compound	450 mμ	500 mμ	550 mμ
Rhodopsin	34	25	11
Isorhodopsin	17	17	14
Metarhodopsin	49	58	75

Hubbard & Kropf (1958) were able to take advantage of this difference in absorption spectrum between cattle rhodopsin and metarhodopsin to demonstrate the photoisomerization of the chromoproteins. Rhodopsin solutions at $-20°$ were irradiated with monochromatic light (half band-width 11 mμ) of 450, 500 and 550 mμ. The shortest wavelength band is absorbed more strongly by metarhodopsin than by rhodopsin; the longest wavelength band has the opposite effect. Three different pseudo-equilibria were obtained by such irradiation (see Table 1). The 450 mμ light gave more rhodopsin, as would be predicted from the photoisomerization theory, since it would be preferentially absorbed to isomerize the metarhodopsin back to rhodopsin; the 550 mμ light did the opposite. It appears that the action of light on cattle rhodopsin is much the same as on squid rhodopsin in acid solution, except that the isomerization is not so specific, some isorhodopsin being formed. (If artificially prepared isorhodopsin is used as the starting material instead of rhodopsin, essentially the same pseudo-equilibrium is formed.)

Hubbard *et al.* (1959) repeated at $-65°$ similar operations on cattle rhodopsin and on invertebrate rhodopsins (Kropf *et al.* 1959), and observed the same phenomenon as at $-20°$. The experiments at $-65°$ involve only rhodopsin, isorhodopsin and lumirhodopsin; they are restricted to the truly photochemical reaction of visual pigments. The action of light is the same on cattle rhodopsin as on squid rhodopsin: it reversibly converts the 11-*cis*-retinene portion of the visual pigment into a steady-state mixture of isomeric chromoproteins.

The exact differences between lumi- and meta-rhodopsin are not clear, but two hypotheses have been put forward. Hubbard *et al.* (1959) postulate that the transformation of lumi- into meta-rhodopsin involves a change in the configuration of the protein opsin, and that no further isomerization of the chromophore occurs. It has been shown that the opsin portion of squid rhodopsin reacts differently when changed to metarhodopsin (Hubbard & St George, 1958). Hubbard *et al.* (1959) suggest that the polypeptide chain of opsin is stabilized in one configuration by the 11-*cis* chromophore in rhodopsin, i.e. one function of the chromophoric group is to hold the protein in the right shape. When the retinene is isomerized, the chromophore pulls away from the protein (see Fig. 2), which becomes unstable. It cannot rearrange itself in the experimental conditions used—a glycerol glass at $-65°$; only when this becomes more fluid can the protein change to a more stable shape. This, Hubbard *et al.* (1959) suggest, is the lumi- to meta-rhodopsin step.

In agreement with this hypothesis Hubbard *et al.* (1959) report that although rhodopsin and lumirhodopsin are readily interconverted by light at $-65°$, as are rhodopsin and metarhodopsin at $-20°$, irradiation of metarhodopsin at $-65°$ does not yield rhodopsin. Hubbard *et al.* (1959) postulate that low temperature blocks the rearrangement of opsin in both directions. The action of light on visual pigments, as proposed by Hubbard and co-workers, is summarized in Fig. 9.

Hagins (1957) independently put forward a scheme somewhat similar, but differing in that he postulated lumirhodopsin (in the newer definition of Hubbard & Kropf, 1958) to be a complex mixture of stereoisomeric lumirhodopsins, rather than predominatingly all-*trans*, as suggested by Hubbard (1958*b*) and Hubbard & Kropf (1958). One or more of the isomers in the mixture might be stable only at low temperatures and isomerize to the more stable all-*trans* form on warming. This views the lumi- to meta-rhodopsin step as a change in the configuration of the chromophore group and does not invoke an alteration in the protein. The results of Wulff *et al.* (1958) with flash photolysis of rhodopsin solutions can be interpreted as indicating that a quite complex isomerization mixture is formed, but they seem to be compatible with the hypotheses of both Hagins (1957) and Hubbard *et al.* (1959).

The experimental approach to the configuration of the chromophores of lumi- and meta-rhodopsin is beset with difficulties, and the results obtained are often hard to relate to physiological conditions. Hubbard & Kropf (1959) have evidence which suggests that the rigid glycerol glass may not only prevent rearrangement of the opsin, but also restrict isomerization of the retinenes when combined with the proteins. Dr W. A. Hagins (personal communication) has pointed out that, given this

assumption, it is possible to advance an explanation in terms of isomerization of the chromophores alone for the inability to re-form rhodopsin from metarhodopsin at $-65°$. Yet to emphasize the hindrance to isomerization of the rigid medium is to view lumirhodopsin partly as an artifact when studied at low temperatures. It is also probable (see the work of Adams *et al.* 1958) that the isomeric constitution of some lumi- and meta-rhodopsins depends on the intensity of irradiation and the rate at which metarhodopsin hydrolyses. The all-*trans*-chromo-

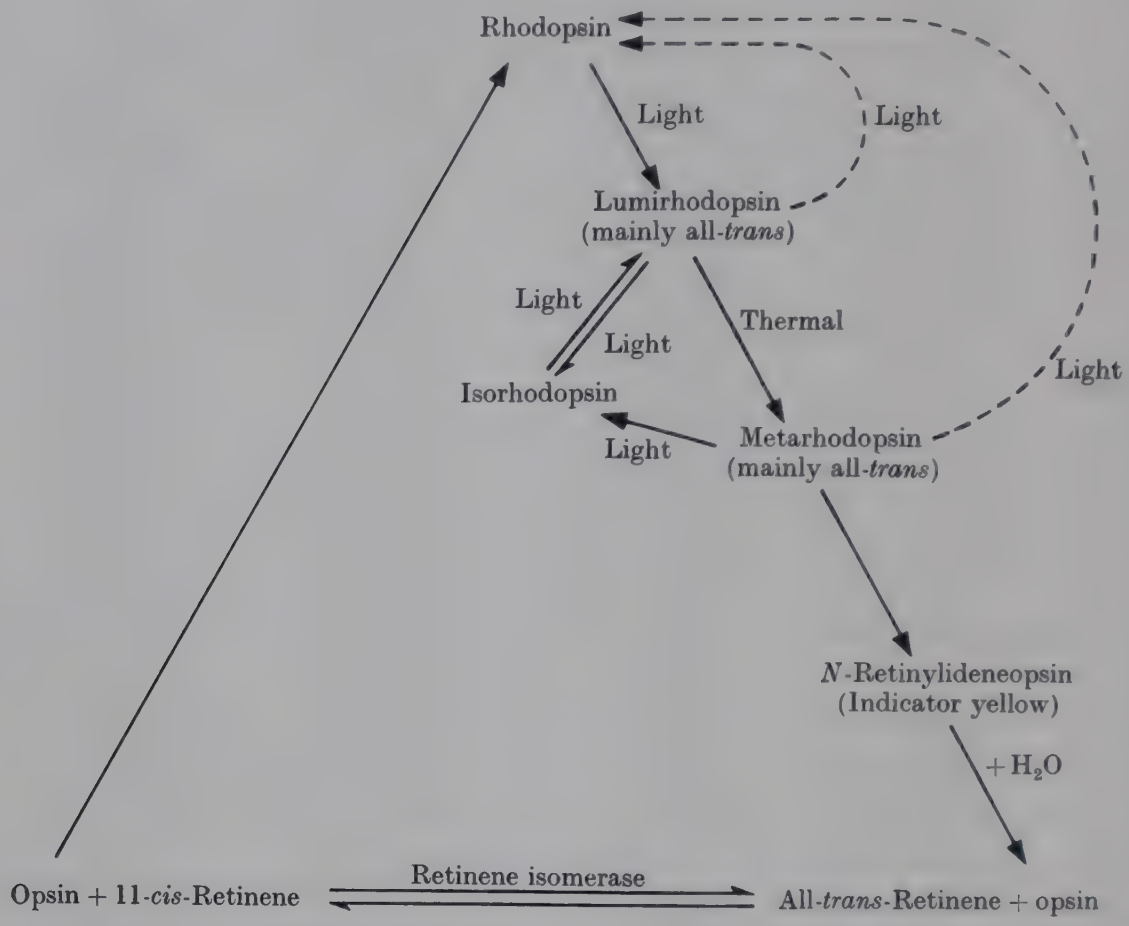


Fig. 9. Action of light on visual pigments.

proteins of many species normally break down quickly; stabilization by low temperature raises the probability of their being reisomerized, beyond what would occur at physiological temperatures. The basic dilemma in studying lumi- and (vertebrate) meta-rhodopsins is that the only conditions in which they can be studied experimentally are such as to increase the likelihood of 'unnatural mixtures' being formed. It is difficult to distinguish between rival hypotheses and doubtful whether any one hypothesis holds for all conditions of illumination (see Adams *et al.* 1958).

It is not easy to predict what should (or could) happen when retinene is isomerized in the chromoproteins. Although much *in vitro* work on the photoisomerization of retinene has been discussed previously, the

isomerized products differ qualitatively and quantitatively with variations (*a*) in the medium, (*b*) in the compounds with which retinene is combined (see above), and (*c*) perhaps in the pH (Hubbard & St George, 1958). Experiments on retinene or its simple compounds *in vitro* may bear little relationship to the isomerizations occurring in the retina, which seem to be more selective (cf. the absence of isorhodopsin from retinas; Wald, 1957). On the other hand, the spectroscopic changes observed in the intact retina are much more complex and harder to interpret than are those in solutions of visual pigments (Hagins, 1957).

Although the action of light alone on rhodopsin seems to be limited to a simple isomerization of the chromophoric group, this sets off a complex series of events resulting in the transmission of the nervous impulse up the optic nerve. It is not known how this impulse is generated, but Hubbard & Kropf (1958) believe that it must arise in going from rhodopsin to metarhodopsin, since invertebrate metarhodopsin is stable in the eye, and bleaching does not normally occur. It is tempting to associate the postulated protein change in the lumi- to meta-rhodopsin step with the initiation of a process leading to the generation of the nervous impulse.

In vertebrate eyes, where metarhodopsin is unstable, Hagins (1956) found that the visual pigment bleached (i.e. went past the metarhodopsin stage) before any electrical response of the retina to a flash could be detected. Even so, to assume that both vertebrate and invertebrate pigments operate in the same mechanism is reasonable but not absolutely safe, since there are differences between vertebrate and invertebrate eyes.

Furthermore, it is not quite certain that cephalopod metarhodopsins are wholly analogous to vertebrate metarhodopsins. Cephalopod metarhodopsins are pH indicators (vertebrate and lobster metarhodopsins show no alkaline form); they have much higher molecular extinction coefficients; they have an absorption maximum at longer wavelengths, with a wide band (Hubbard *et al.* 1959; Kropf *et al.* 1959). Hubbard recognizes these differences, but prefers to stress the similarities. She is probably right to do this, but the discrepancies should not be overlooked.

These reservations are minor and do not detract from the outstanding achievements of Wald, Hubbard and the Harvard school in discovering and working out so thoroughly the isomerization cycle of retinene and rhodopsin. There are still obscure points; in particular many of the spectroscopic observations of Hubbard *et al.* (1959), Kropf *et al.* (1959) and Hubbard & Kropf (1959) are hard to explain. The handicap here is a lack of firm knowledge why 11-*cis*-retinene on uniting with opsin gives an absorption peak near 500 mμ.

This review has concentrated on rhodopsin, the retinene$_1$-pigment of

vertebrate rods, and of some invertebrates. Porphyropsin, the rod pigment based on retinene$_2$, has not been studied so thoroughly, but appears to behave in the same way (Wald, 1953, 1955). More important to man and many other species are the cone pigments, but less chemical work has been done on them, as it is difficult to extract enough cone pigment from eyes. Wald, Brown & Smith (1955) succeeded in preparing iodopsin, a cone pigment from chicken eyes, with an absorption maximum near 560 mμ (Fig. 10). It showed the same overall isomerization cycle as did rhodopsin, being formed from cone opsin (or

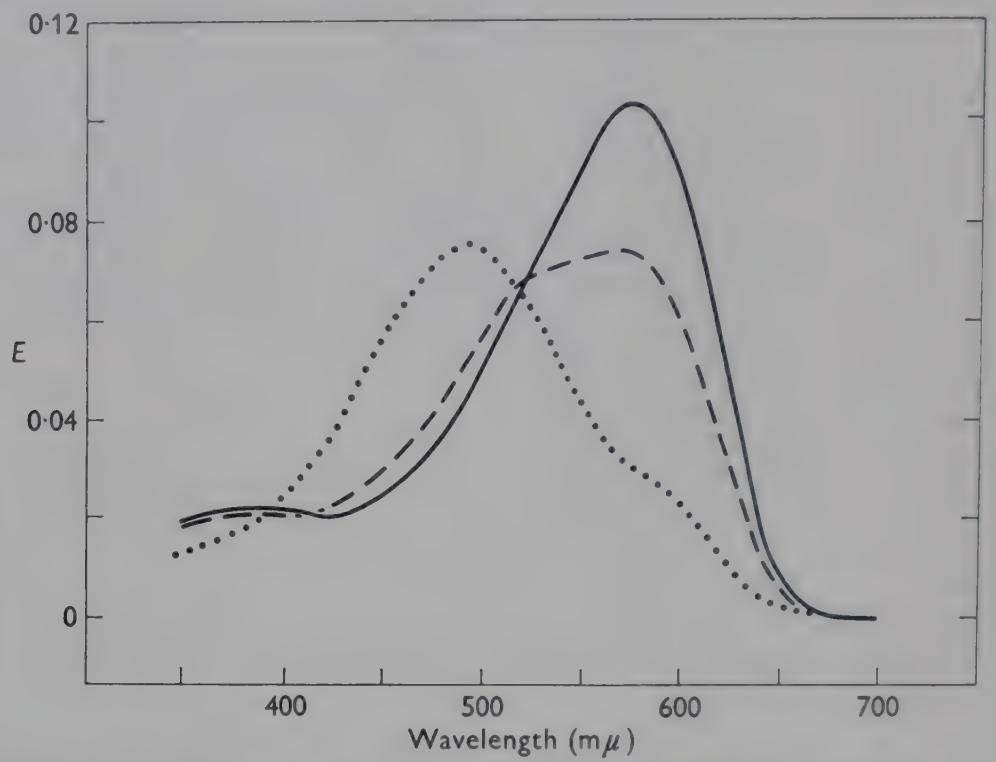


Fig. 10. Interconversions of iodopsin and metaiodopsin. Absorption spectra of iodopsin in glycerol water (2:1, v/v) (+ digitonin) at $-38°$ (——); irradiated with light of 610 mμ (mainly metaiodopsin with some iodopsin) (. . .); this product now irradiated with light of 450 mμ (a large part converted back into iodopsin) (– – –) (Hubbard & Kropf, 1959).

photopsin) and 11-*cis*-retinene. Light bleached it to photopsin and all-*trans*-retinene. Hubbard & Kropf (1959) have investigated iodopsin in the same way as rhodopsin and have shown that it behaves very similarly. Irradiation below $-45°$ with long-wavelength light (610 mμ) produces some lumi-iodopsin with $\lambda_{max.}$ near 525 mμ; allowed to warm up a little, this gives metaiodopsin ($\lambda_{max.}$ near 500 mμ) which hydrolyses in the dark above $-30°$ to give mainly all-*trans*-retinene and photopsin. Since there is a big difference in the absorption spectra of iodopsin and metaiodopsin, it is possible by using lights of suitable wavelengths to irradiate one with relatively little effect on the other. Light of 610 mμ at a suitable temperature converts almost all the iodopsin into metaiodopsin; subsequent irradiation at 450 mμ converts much of the meta-

iodopsin back to iodopsin. From Fig. 10 it can be seen that iodopsin shows, much more clearly than is possible with vertebrate rhodopsin, the isomeric changes brought about by light.

Other visual pigments are known in addition to rhodopsin and the rod and cone pigments of vertebrates. Goldsmith (1958) has found in the honey-bee a photosensitive pigment with $\lambda_{max.}$ near 440 mμ, based on retinene. Fisher & Kon (1959) have reviewed work done on the visual pigments of crustacea, which indicates that they, too, are probably retinene pigments. No report has yet appeared on the configuration of the retinene in these newly discovered pigments, but it seems probable, particularly in crustacea, that it will be the 11-*cis* form which has been found in all other visual pigments investigated, and that light will isomerize it to the all-*trans* configuration.

This is the device widespread in Nature for detecting radiation in what we know as the visible region of the spectrum. The 11-*cis*-retinene which is very readily isomerized by light, is coupled with an opsin in some way not yet understood, which shifts the absorption peak of the retinene to much longer wavelengths. Light striking the visual pigment converts the chromophore into a steady-state mixture of stereoisomers. Some of these are unstable (lumi-pigments), as the shape of their chromophoric groups does not allow a stable configuration to be formed with the protein. Thermal reactions are set in train, leading to the initiation of a nervous impulse.

Experiments both *in vitro* and *in vivo* show this reversible photo-isomerization in all visual pigments studied. Light cannot only break down visual pigments, it can also re-form them from the early photo-products. To what extent do these short cuts (shown as dotted lines in Fig. 9) operate in the eye? Their importance in vertebrates will depend on the light flux and on the rate in the eye at which the reactions proceed between lumirhodopsin and *N*-retinylideneopsin (or equivalent derivatives of other pigments) (see Adams *et al.* 1958). In invertebrate eyes these 'short cuts' are probably important. Such eyes appear to show a fine example of economy. Light, by isomerizing the 11-*cis*-retinene chromophore to the all-*trans* form, presses the trigger which results in the production of the nervous impulse; it can also, by putting energy into the all-*trans*-chromophore, isomerize it back to the 11-*cis* isomer, which is at a higher energy level.

The common steric analogy in biochemistry is that of the lock and key: molecules must be of the right shape to fit together and react. Visual pigments show the lock-and-key phenomenon—retinene must be bent at the right place to unite with the opsins—but there is a more important steric feature: the isomerization of the molecule is the mechanism by which it operates. There can be few *cis–trans* changes

which make so constant and so vivid an impact on us as does that of retinene. For it is because retinene is isomerized by light that we can see.

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DISCUSSION OF PAPER BY PITT AND MORTON

J. W. Cornforth (*National Institute for Medical Research, Mill Hill*): The very large shift in the wavelength of maximum absorption when retinenes combine with opsin suggests some kind of halochromism, and this would be consistent with ease of dissociation. Has any study been made of the absorption spectrum of retinene in the presence of Lewis acids?

G. A. J. Pitt: Free retinene with antimony trichloride (which is believed to induce halochromism) shows an absorption maximum near 665 mμ, well on the long-wavelength side of rhodopsin's or even iodopsin's peak; concentrated mineral acids react similarly. Retinene, however, is joined by its aldehyde group to an amino group to form a Schiff's base in rhodopsin. Azomethine derivatives of retinene do show, with antimony trichloride or strong acids, absorption maxima near 500 mμ, with other spectroscopic features similar to those of rhodopsin (see Pitt *et al.* 1955). Although the comparison of rhodopsin and these artificial chromogens should not be pressed far, these findings have directed attention to the potentialities of Schiff's bases in the chromophores of visual pigments, a theme subsequently developed by Hubbard (1958*a*) and Kropf & Hubbard (1958).

STERIC FACTORS IN ENZYME ACTION: HYDROLYTIC ENZYMES

By E. C. WEBB

Department of Biochemistry, University of Cambridge

Considerable attention has been focused in recent enzymological work on the chemical structure of the 'active centre'. A number of different methods have been recently developed for such work: for example chemical attack on particular amino acids, or 'labelling' of the active centre with an isotopically labelled inhibitor and subsequent determination of amino acid sequences containing the label. Nevertheless, one of the older methods of examining the active centre, namely a detailed study of enzyme specificity, can probably still yield a great deal of information about the probable nature and orientation of the catalytic groupings.

Any attempt to generalize about the optimum requirements in a substrate for attack by a particular enzyme is concerned, in the widest sense, with 'steric factors', since the shape of the substrate and its 'fit' to the active centre play a great part in determining those requirements. However, relatively more information about steric, as opposed to purely chemical, factors, is given if the substrate is asymmetric. It is in this narrower sense that I shall interpret 'steric factors' for the purposes of this review: taking 'steric factors in enzyme action' to mean a study of stereospecificity, or the ability of an enzyme to attack selectively one of a pair of stereoisomers.

Hydrolytic enzymes, which form about one-third of known enzymes, include a large number of enzymes of very low specificity. They are thus particularly suitable for the kind of study I have just described. Even if the typical substrate is a symmetrical molecule, it is often possible to introduce asymmetric groupings into this substrate without loss of activity, and then study the enzyme activity towards the resolved enantiomorphs. Conversely, with such enzymes of low specificity the additional information obtained by studying asymmetric substrates may be essential before useful generalizations about combining structures at the active centre can be made.

KINETIC CONSIDERATIONS

It is now generally accepted that enzymic catalysis is at least a two-stage process, proceeding through an intermediate enzyme–substrate (ES) complex. It is clear that steric factors might be important in both

stages, in the formation of the intermediate complex as well as in ensuring its breakdown to yield the products of the catalysed reaction. Quantitative studies of the relative efficiency of an enzyme on a series of substrates should if possible be carried out so as to distinguish the contribution of the two stages.

Many of the statements of enzyme specificity in the literature merely record the relative rates of reaction of a number of substrates, all tested at the same standard substrate concentration. Differences between one substrate and another might be due to differences in combining power (affinity) or to differences in rate of reaction of the ES complexes. This will be true even if the standard concentration has been chosen to saturate the enzyme with the substrate first investigated, since if a second substrate has an appreciably lower affinity it will not saturate the enzyme at the standard concentration and the rate of reaction may be lower due to this factor alone. To be of real value comparisons of substrates should be made by measuring both reaction velocity (V) and Michaelis constant (K_m) for each substrate. This, of course, involves a considerable amount of labour, since complete Michaelis–Menten curves must be obtained for each separate substrate.

Failure to distinguish between changes in V and K_m has been responsible in the past for some confusing and even contradictory statements about the stereochemical specificity of enzymes. This is particularly true of ali-esterases, as is explained below.

On the simplest interpretation of Michaelis–Menten kinetics, K_m and V can be taken as a measure of the two stages, formation and breakdown of the ES complex. However, under some circumstances K_m may not approximate to K_s, the reciprocal of the affinity of the enzyme for its substrate, but may have a more complex kinetic meaning, involving additional steps beyond the formation of the primary complex (the non-identity of K_m and K_s has been confirmed in a number of cases, particularly by the work of Chance; for a discussion see Dixon & Webb, 1958). Thus an accurate impression of the stereochemistry of combination with the active centre is not necessarily given by a study of the K_m values obtained with asymmetric substrates. For this reason it is useful to extend a study of steric factors in enzyme action to include competitive inhibitors. The value of K_i for a competitive inhibitor is a true (reciprocal) measure of combining power, since K_i is a dissociation constant uncomplicated by any kinetic component. In a number of cases discussed below the affinities of an enzyme for the two enantiomorphs of an asymmetric competitive inhibitor have been determined. Apart from the fact that K_i has a more clearly defined meaning than K_m, such measurements with inhibitors are a useful way of extending the investigation of the influence of structure on affinity to more highly

specific enzymes, for which it may not be possible to devise substrates containing asymmetric structures.

ESTERASES

The ali-esterases and lipases of animal tissues are enzymes of very wide specificity, hydrolysing almost any compound containing an ester bond, although at greatly varying rates. Dakin (1904, 1905) first showed that when an ester of an acid containing an asymmetric carbon atom was hydrolysed by an enzyme, there was a preferential attack on one antipode. Thus when racemic ethyl mandelate was *partially* hydrolysed by liver 'lipase', the mandelic acid produced was dextrorotatory. After a lapse of two decades Dakin's observations were confirmed and extended by Willstätter and Rona, and still later by Bamann in Munich and Ammon in Berlin. The subject has been reviewed by Ammon & Jaarma (1951). It should be stressed that almost all these experiments were carried out with crude enzymes, usually extracts of acetone-dried tissues, and have not so far been repeated with the pure enzymes now available.

Table 1. *Action of esterases on* DL-*esters* (*after Rona & Chain*, 1933)

+ and − signs show the rotation of the ester preferentially removed from the racemate.
· indicates 'not studied'.

	Enzyme preparation from							
	Pancreas		Liver		Stomach		Serum	
Ester	Human	Pig	Pig	Carp	Pig	Dog	Guinea pig	*Aspergillus*
Methyl mandelate	−	−	+	·	·	·	−	+
Ethyl mandelate	−	−	+	−	+	+	·	+
Methyl phenyl-chloroacetate	·	−	−	·	·	+	·	−
Methyl tropate	·	+	−	·	+	·	·	−
sec.-Butyl *n*-butyrate	·	−	−	·	·	·	·	·

Not all the esterases examined showed the same optical preference. Table 1, taken from Rona & Chain (1933), shows the rotation of the antipode preferentially attacked when crude enzyme preparations hydrolysed DL substrates. Taking the data for the pig, it would appear that pancreatic lipase preferentially attacks (−)-methyl D-mandelate, while liver esterase prefers (+)-methyl L-mandelate. However, when Rona & Ammon (1927) used pure antipodes as substrates, the opposite result was obtained for the liver enzyme (Table 2). Both pancreatic and liver enzymes hydrolyse methyl D-mandelate significantly faster than its enantiomorph. Weber & Ammon (1929) showed that the explanation for the apparent discrepancy is that the affinities as well as the maximum

velocities are different for the two isomers with liver esterase, although not with pancreatic lipase (Fig. 1). With pig-liver esterase the affinity for (+)-methyl L-mandelate is seven times that for the D-mandelate, although the maximum velocity of hydrolysis of D-mandelate is 1·75 times that of the L-isomer. The higher affinity causes the preferential hydrolysis of the L(+)-ester in a DL mixture. Closeness of fit is evidently

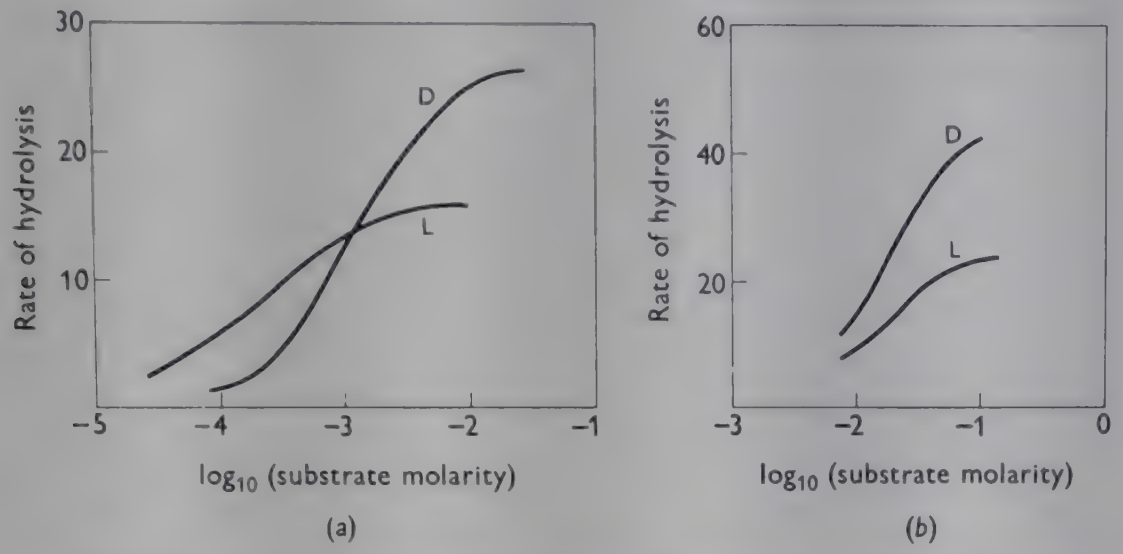


Fig. 1. Hydrolysis of optically active methyl mandelates at varying substrate concentrations: (*a*) by an extract of acetone-dried pig liver; (*b*) by an extract of acetone-dried pig pancreas (after Weber & Ammon, 1929).

not necessarily advantageous for maximum catalytic efficiency. Mandelic esters of the L configuration combine more readily with liver ali-esterase to give a more stable complex, which is less catalytically active and is hydrolysed more slowly.

Table 2. *Action of esterases on methyl* D-, L-, *and* DL-*mandelate* (*after Rona & Ammon*, 1927; *Rona* et al. 1930*a*)

Initial rates of hydrolysis are expressed for each enzyme relative to the rate with D-ester.

	D (−)	L (+)	DL
Pig pancreatic lipase	1·0	0·65	0·75
Pig liver esterase	1·0	0·59	0·54
'Takalipase' from *Aspergillus oryzae*	1·0	0·49	.

The relative rate of hydrolysis of the two isomers varies with the substrate concentration not only because of differences in affinity, but also because of differences in the inhibitory effect of high substrate concentrations. Fig. 2, from Bamann (1929), shows the effect of substrate concentration on the hydrolysis of ethyl D- and L-mandelate by liver ali-esterase from a number of species. Since some of the curves cross, there is an actual change of preference from one isomer to the other. Surprisingly, this also applies to the hydrolysis of DL-mandelic

esters by human and rabbit-liver ali-esterases, which produce laevorotatory mandelic acid at low substrate concentrations and dextrorotatory acid at higher concentrations (Bamann, 1929; Ammon & Geisler, 1932). Murray (1933) has shown that with an equimolar mixture of two substrates with different values of V and K_m, the *ratio* of the rates of disappearance of the two substrates should be independent of the concentration:

$$v_a/v_b = (V_a \cdot K_b)/(V_b \cdot K_a) \quad \text{(see Dixon \& Webb, 1958).}$$

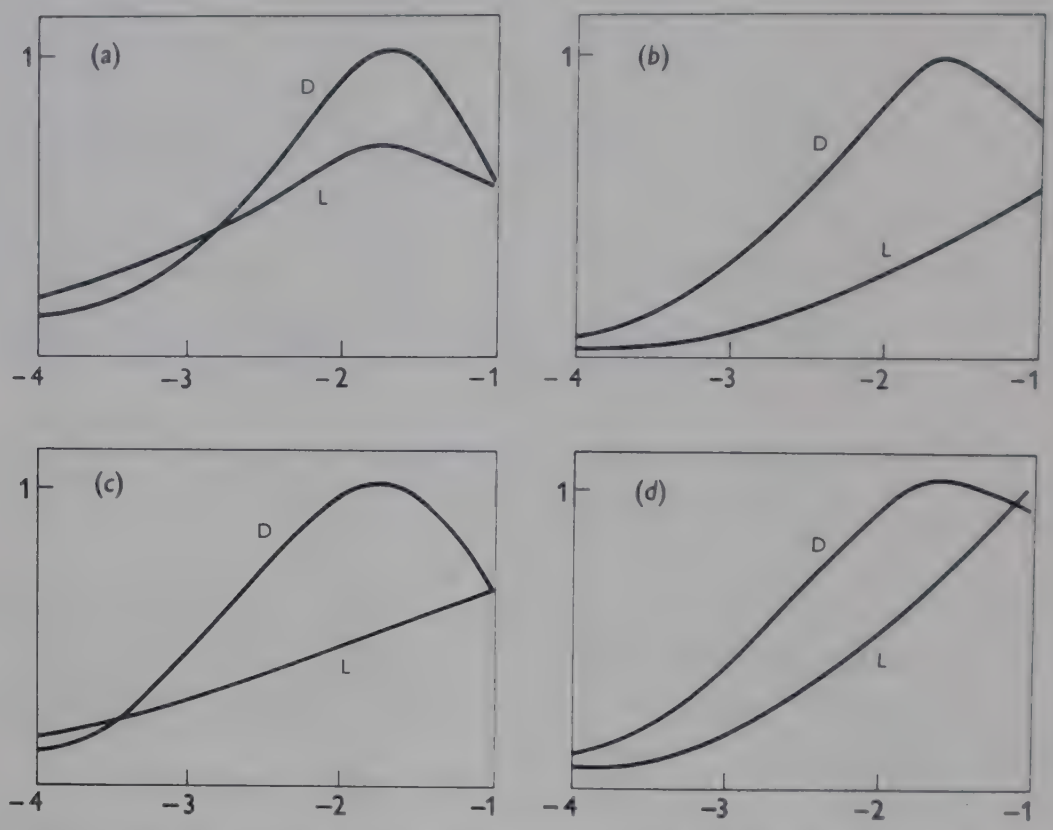


Fig. 2. Hydrolysis of optically active ethyl mandelates by liver ali-esterase from various species at varying substrate concentration: (*a*) sheep; (*b*) rabbit; (*c*) dog; (*d*) man. Ordinates show relative activity; abscissae $\log_{10}$ (molarity of substrate) (after Bamann, 1929).

A more complex explanation for the inversion with concentration in the hydrolysis of DL-mandelates has been given by Schwab, Bamann & Laeverenz (1933); the rate of breakdown of the ES complexes is assumed to be dependent on the substrate concentration, perhaps because of the varying alcohol concentration produced.

The stereospecificity of liver ali-esterases can be influenced in a number of ways, e.g. by the addition of alkaloids (Bamann & Laeverenz, 1930) or ethanol (Bamann & Laeverenz, 1931). A detailed investigation by Ammon & Fischgold (1931) of the effect of strychnine on human ali-esterase showed that it had no effect on the affinity of the enzyme for either isomer of methyl mandelate, but it increased V for methyl

D-mandelate by about 50 %. Thus strychnine considerably increases the stereochemical specificity of the enzyme.

The relationship between configuration and affinity for liver ali-esterase has also been investigated by the use of optically active inhibitors (Murray & King, 1930). The hydrolysis of ethyl butyrate or propionate by sheep-liver esterase was inhibited 4–5 times more strongly by the L(−)-forms of methyl-*n*-hexylcarbinol, methylphenylcarbinol and methyl-*β*-phenylethylcarbinol than by the D forms. With rabbit-liver enzyme there was little difference between the two forms. These results are qualitatively in agreement with the differences in affinity for L and D substrates for the two species, shown in Fig. 2.

When an enzyme shows stereospecificity in a reaction in which the asymmetric centre is destroyed, it will, in the reverse reaction, carry out an 'asymmetric synthesis', producing an optically active product. Such asymmetric syntheses have been demonstrated with esterases for the formation of esters of lactic acid (Rona & Ammon, 1930), *sec.*-butanol (Rona, Ammon & Werner, 1930*b*), and a series of related carbinols and acids (Rona & Chain, 1933).

As might be expected, considerable differences have been found in the rate of synthesis of esters of *cis–trans* isomers. Thus Fabisch (1931) found that pancreatic preparations formed *n*-butyl maleate twice as rapidly as *n*-butyl fumarate. On the other hand Steensholt (1943) reported that ethyl fumarate was hydrolysed by liver esterase considerably more rapidly than was ethyl maleate.

Cholinesterases. The cholinesterases are much more specific than the ali-esterases discussed above; from information about the effect of changes in the structure of the substrate, as well as kinetic studies of the effect of pH and inhibitors, a detailed picture of their active centre and mode of action has been evolved (see, for example, Adams, 1949; Adams & Whittaker, 1949; Sturge & Whittaker, 1950; Mounter & Whittaker, 1950; Nachmansohn & Wilson, 1951). There are two groups of cholinesterases: acetylcholinesterase with high affinity for acetylcholine, but depressed rates of hydrolysis at higher substrate concentrations, and a high specificity for the acetyl grouping (butyrylcholine is hydrolysed at 1–2 % of the speed of acetylcholine); and cholinesterase (earlier called 'pseudo-cholinesterase') with a much lower affinity for acetylcholine and a preference for larger acyl groups (butyrylcholine is hydrolysed up to 2·5 times as fast as acetylcholine). The special susceptibility of choline esters, at any rate for acetylcholinesterase, has been explained in terms of an anionic specificity site which combines with the positively charged trimethylammonium group. The active centre of acetylcholinesterase is believed to possess a bifunctional 'esteratic site', which actually catalyses the hydrolysis, as well as the

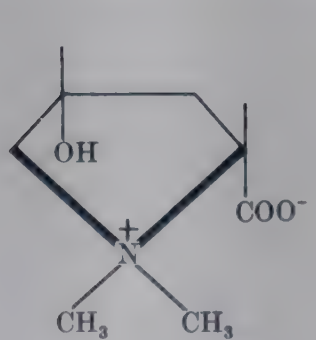
anionic site, and the distance between these sites is an important factor in determining the specificity of the enzyme. The shape of the substrate is almost as important as the positive charge, and simple aliphatic esters are hydrolysed, the velocity increasing as their structures approximate in shape to acylcholines. The closest analogue, 3:3-dimethylbutyl acetate, is hydrolysed by cholinesterase at 35 % of the rate of acetylcholine and by acetylcholinesterase at 60 % of the acetylcholine rate.

Most of the substrates of the cholinesterases which have been examined are symmetrical. However, acetyl-β-methylcholine, which has been used as a 'specific substrate' for acetylcholinesterase, being only very slowly attacked by cholinesterase, has an asymmetric carbon atom and can be resolved. Glick (1938) showed that the (−)-form is not hydrolysed by horse-serum cholinesterase, while the (+)-form and the (±)-compound are hydrolysed at the same maximum rate, which is 1·6 % of that of acetylcholine. Thus the (−)-form cannot combine with the enzyme either as a substrate or inhibitor.

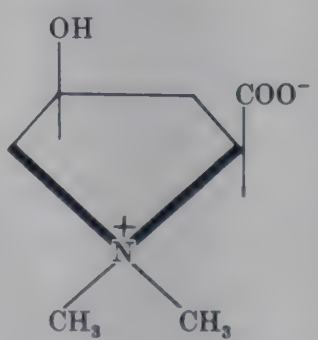
In a number of cases acetylcholinesterase shows stereospecificity to inhibitors. L-α-Amino acids are weak inhibitors, while D-amino acids are ineffective (Bergmann, Wilson & Nachmansohn, 1950). With the compound $(CH_3)_2N \cdot CH(CH_3) \cdot CH_2 \cdot NC_5H_5$, the (+)-form has 4 times the inhibitory power of its antipode (Friess, Whitcomb, Hogan & French, 1958).

A study of some *cis–trans* isomers has provided useful confirmation of the idea of a critical distance between the groups which must combine with the anionic and esteratic sites (Baldridge, McCarville & Friess, 1955; Friess & Baldridge, 1956). The *cis* and *trans* isomers of (±)-2-trimethylamino*cyclo*hexanol and (±)-2-trimethylamino*cyclo*pentanol were compared as inhibitors of electric-eel acetylcholinesterase, and the corresponding acetates were compared as substrates. The results are shown in Table 3. They suggest a distance of about 2·5 Å between the anionic and esteratic sites of acetylcholinesterase.

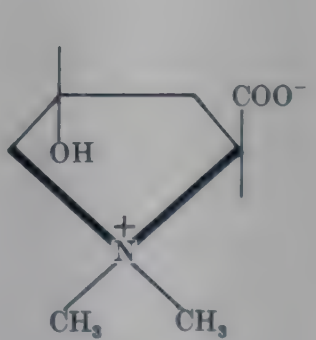
The two natural betaines D-turicine and L-turicine do not inhibit acetylcholinesterase, while the *trans* isomer L-betonicine has a K_i of 85μM (Friess, Patchett & Witkop, 1957).



D-Turicine

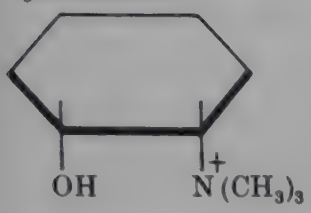
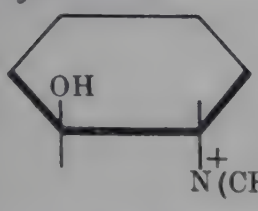
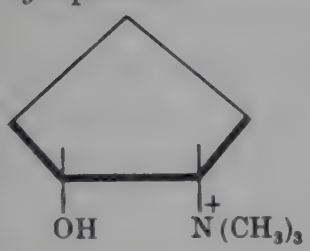
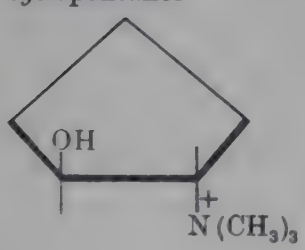


L-Turicine



L-Betonicine

Table 3. *Some* cis–trans *isomers as inhibitors and substrates of acetylcholinesterase*

Alcohol tested	Inhibitory power of alcohol $10^4 K_i$	Activity of corresponding acetate as substrate: Concn. for optimum velocity (mM)	Activity of corresponding acetate as substrate: Rel. max. velocity	O – N distance in alcohol (Å)
Choline	4·5	3·3	1·0	2·3
cis-(±)-2-Trimethylamino-*cyclo*hexanol 	1·1	1·8	1·14	2·5–2·9
trans-(±)-2-Trimethylamino-*cyclo*hexanol 	2·1	2·1	1·06	2·9–3·7
cis-(±)-2-Trimethylamino-*cyclo*pentanol 	0·75	2·95	1·43	2·51
trans-(±)-2-Trimethylamino-*cyclo*pentanol 	0·89	3·07	1·07	3·45

Clearly the absolute configuration on C-2 is not important, since neither D- nor L-turicine is active. Inhibition presumably depends on the close approach of the –OH and $-N(CH_3)_2^+$ groups to the enzyme surface, and this will be blocked by the bulky $-COO^-$ group in both turicines. In the *trans* compound the carboxyl radical will not interfere.

GLYCOSIDASES

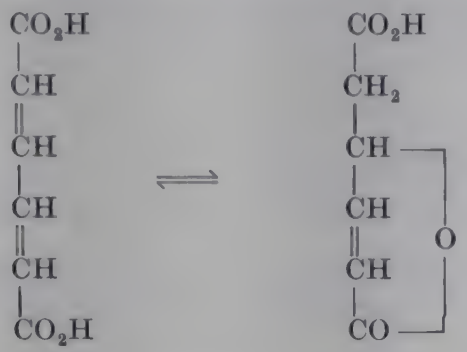
Glycosidases form a marked contrast with esterases as a group, since they are in general highly specific for the sugar moiety (see the review by Gottschalk, 1950). Steric considerations must be of primary importance here, since all the determining groups are either hydroxyl groups or hydrogen atoms, so that the specificity must be determined by the pattern in which they are arranged. Of course, in these substrate molecules with many asymmetric centres, changes in the configuration of any of them have a marked effect on the chemical properties of the molecule as a whole. In most cases such changes in the substrate of a glycosidase make it resistant to the enzyme. Gottschalk says: 'There is no....case known where a glycosidase tolerates a change in the configuration at one or more of the carbon atoms 1, 2, 3, 4 of the glycon.' Thus a glycosidase is specific not only for glycosides of a particular sugar, but also for either α or β configuration at the reducing group; α-D-glucosidase, β-D-glucosidase and α-D-mannosidase, for example, are separate enzymes with no common substrates.

A large volume of data has been accumulated about the effect of small changes in glycosidase substrates, but unfortunately most of it has been obtained with rather crude enzymes by measurement of the first-order velocity constant, v_0, at low substrate concentration, which is equal to V/K_m (Dixon & Webb, 1958). Thus it is impossible to distinguish between effects of affinity and changes in catalytic efficiency; the former are probably often the more important. Thus the loss of activity towards β-D-glucosidase when the hydroxyl groups on C-2, C-3 or C-4 are inverted or substituted is presumably due to loss of the combining structure necessary for the 'fit' of the glycoside to the active centre. Some replacements of the terminal $-CH_2 \cdot OH$ group can be made; for example, β-D-xylosides are hydrolysed at 0·55 % of the rate of hydrolysis of β-D-glucosides; but the rate falls off sharply as the volume of the substituting group is increased (Helferich, Grünler & Gnüchtel, 1937).

Most glycosidases are relatively unspecific for the aglycone. Changes in the aglycone probably affect the rate by chemical rather than by purely steric means. Thus Nath & Rydon (1954) showed that in a series of aryl-β-D-glucosides, introduction of electron-attracting groups which made them more susceptible to alkaline hydrolysis increased both affinity for and velocity of breakdown by β-D-glucosidase.

Lactonases. The lactonases which have been discovered comparatively recently seem to have the same kind of rigid structural requirements as the glycosidases. Brodie & Lipmann (1955) showed that gluconolactonase from yeast would only attack lactones with the same configuration as D-glucono-δ-lactone, out of a large number examined.

Carboxymethyl-γ-hydroxy*iso*crotonolactonase will hydrate only the (+)-butenolide; the (−)-lactone is not attacked, nor does it inhibit the reaction (Sistrom & Stanier, 1954). On the other hand, in the formation of the butenolide by the muconate-lactonizing enzyme,



cis–cis-muconate is converted into (+)-carboxymethyl-Δ^{α}-butenolide, and *cis–trans*-muconate is converted (at 0·02% of the rate with the *cis–cis* compound) into the (−)-butenolide. *trans–trans*-Muconate is not attacked at all. Sistrom & Stanier point out that this shows that the enzyme can discriminate between the two component bonds of the $\gamma\delta$ double bonds of the muconates, and between the two δ-hydrogen atoms of the lactone.

PEPTIDASES

The use of synthetic peptides as test substrates, initiated by Bergmann, has led to a fairly clear picture of the substrate requirements of the peptidases. The general principles have been ably set out in a number of places (e.g. Neurath & Schwert, 1950) and will not be repeated in detail here. Specificity is primarily related to the residues adjacent to the bond hydrolysed: with endopeptidases the amino acids on both sides of the attacked peptide link are important, while with the exopeptidases it is only the terminal amino acid residue (*N*-terminal or *C*-terminal for aminopeptidases or carboxypeptidases respectively) which is particularly important.

Stereochemical results confirm this general picture. The common peptidases are L-specific, and D-amino acid residues in the determining positions mentioned prevent hydrolysis or reduce it to perhaps one-thousandth of the normal rate. On the other hand, D-amino acids can often be introduced into other positions in the peptides without great loss of susceptibility. Four enzymes will be discussed to illustrate these points.

Table 4 gives proteolytic coefficients (which are related to the first-order velocity constants (V/K_m)) for the hydrolysis of a number of peptides and amides by a partially purified leucine aminopeptidase (Smith & Polglase, 1949). The requirement for the L configuration is

absolute for the *N*-terminal leucine residue, and even in the next position replacement of L- by D-alanine lowers the rate to one-twentieth, although the lack of positive requirements in this position is shown by the high rates with L-leucineamide and L-leucyl-β-alanine. Presumably there is steric interference with enzyme–substrate binding by the β-methyl group of the D-alanine.

Table 4. *Stereospecificity of leucine aminopeptidase (from Hanson & Smith, 1948; Smith & Polglase, 1949)*

Proteolytic coefficients [for definition see Hanson & Smith (1948), Table 1] for hydrolysis by a partially purified leucine aminopeptidase from pig intestinal mucosa, relative to that for L-leucineamide.

L-Leucineamide	100	D-Leucineamide	< 0·03
Glycineamide	0·32		
L-Leucylglycine	114	D-Leucylglycine	< 0·03
		D-Leucyl-L-tyrosine	< 0·03
L-Leucyl-L-alanine	100	L-Leucyl-D-alanine	4·2
L-Alanyl-L-leucineamide	260	D-Alanyl-L-leucineamide	0·32
L-Leucyl-β-alanine	93		

Robinson, Birnbaum & Greenstein (1953) studied the specificity of a glycyl dipeptidase from kidney, especially with a long series of glycyl dipeptides and dehydropeptides. Some of the results are shown in Table 5, where the figures represent relative *V* values. It will be seen that, in general, the rates for D-peptide, L-peptide and dehydropeptide are of the same order. In cases where the structure of the second amino acid interferes with hydrolysis, e.g. in glycylvaline or glycylisoleucine, similar low rates are obtained with L- and D-peptides, although a considerably higher rate is given with the dehydropeptide. When alanine replaces glycine as the *N*-terminal residue, the configuration of the second residue still has little effect, but D-alanyl peptides are attacked at something like one-thousandth of the rate of L-alanyl peptides.

Table 5. *Stereospecificity of glycyl dipeptidase (dehydropeptidase I) (from Robinson, Birnbaum & Greenstein, 1953)*

Rates of hydrolysis of 0·05 M-substrate (sufficient to produce maximum rates) by a partially purified enzyme from pig kidney, relative to that for glycyl-L-alanine.

Glycyl-L-alanine	100	Glycyl-D-alanine	235	Glycyldehydroalanine	235
Glycyl-L-methionine	270	Glycyl-D-methionine	195		
Glycyl-L-norvaline	185	Glycyl-D-norvaline	140	Glycyldehydronorvaline	95
Glycyl-L-leucine	180	Glycyl-D-leucine	103	Glycyldehydroleucine	86
Glycyl-L-norleucine	162	Glycyl-D-norleucine	170	Glycyldehydronorleucine	245
Glycyl-L-isovaline	72	Glycyl-D-isovaline	44		
Glycyl-L-valine	15	Glycyl-D-valine	20	Glycyldehydrovaline	103
Glycyl-L-isoleucine	3	Glycyl-D-isoleucine	7·5	Glycyldehydroisoleucine	120
Glycyl-L-phenylalanine	175	Glycyl-D-phenylalanine	85	Glycyldehydrophenylalanine	54
Glycyl-L-serine	100	Glycyl-D-serine	120		
Glycyl-L-threonine	32	Glycyl-D-threonine	6		
L-Alanyl-L-alanine	290	D-Alanyl-D-alanine	1·5	L-Alanyl-D-alanine	125
L-Alanylglycine	250	D-Alanylglycine	0·1		

Carnosinase is an enzyme which is stereospecific for the *C*-terminal group, which must be L-histidine (Waldschmidt-Leitz, Ziegler, Schäffner & Weil, 1931; Waldschmidt-Leitz & Kofranyi, 1933; Smith & Polglase, 1949). L-Alanyl-, D-alanyl-, and β-alanyl-L-histidine differ in rate of hydrolysis by a factor of less than two. On the other hand, β-alanyl-D-histidine is not hydrolysed at all.

Carboxypeptidase is a good example of an endopeptidase with rigid requirements about the configuration of the carbon atoms adjacent to both the –CO– and –NH– of the susceptible bond (Neurath & Schwert, 1950). Replacement of either of the L- by D-amino acids in the typical acyl-dipeptide substrates results in complete loss of susceptibility.

The high stereospecificity of proteolytic enzymes has enabled them to be used as tools in the resolution of synthetic amino acids. [Greenstein (1954) has reviewed all the methods used in the resolution of amino acids.] Synthetic reactions have been widely used, e.g. the synthesis of anilides or phenylhydrazines from *N*-acyl-amino acids under the influence of papain. However, the stereospecificity is often far from absolute, and indeed, by using papain, a pure sample of *N*-allyloxycarbonyl-D-leucine phenylhydrazide was prepared from the acyl-D-amino acid (Milne & Stevens, 1950). The degree of resolution achieved in the synthesis of phenylhydrazides with papain depends greatly on the *N*-acyl group (Bennett & Niemann, 1950; Schuller & Niemann, 1951). With acylphenylalanines as substrates, appreciable amounts of D-phenylhydrazides were formed when the acyl group was benzyloxycarbonyl or alkyloxycarbonyl; only with *N*-acetylphenylalanine was the D-isomer completely unreactive and resolution of the racemate complete. These differences are at any rate partly due to differences in the solubility of the products.

Enzymic hydrolysis of substituted racemic amino acids has also been successfully used in resolution. DL-Tryptophan methyl ester was resolved by hydrolysis in the presence of chymotrypsin or trypsin, giving a mixture of D-tryptophan methyl ester and L-tryptophan (Brenner, Sailer & Kocher, 1948). Aminoacylases can also be used, and the degree of resolution attained is very high; with aminoacylase I the maximum rate of hydrolysis of *N*-acetyl-L-methionine is 10,000 times that of the D-isomer, and with 19 other acetyl-D-amino acids hydrolysis cannot be detected at all (Birnbaum, Levintow, Kingsley & Greenstein, 1952).

Very interesting results have been obtained from studies of amino acid derivatives as competitive inhibitors of peptidases. The hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine by carboxypeptidase is competitively inhibited by D-phenylalanine and D-histidine, although the D-isomer of the substrate is not hydrolysed and does not inhibit the

enzyme (Elkins-Kaufman & Neurath, 1948, 1949). At neutral pH L-phenylalanine does not inhibit, although it does so at pH 9 or in the presence of high concentration of orthophosphate or pyrophosphate (Neurath & De Maria, 1950). Even at pH 9, however, K_i for L-phenylalanine has a value nine times that for D-phenylalanine. It would seem that the positively charged α-amino group, in the L configuration, interferes with binding to the enzyme, so that the L-amino acid inhibits only when the charge is suppressed.

Table 6. *Affinities of some pairs of stereoisomers for α-chymotrypsin (after Huang & Niemann*, 1951*a*, *b*; *Huang, MacAllister, Thomas & Niemann*, 1951; *Thomas, MacAllister & Niemann*, 1951)

Where a figure is given in the K_m column, the substance is hydrolysed by α-chymotrypsin; where a figure is given in the K_i column, the substance is a competitive inhibitor.

	K_m (mM)	K_i (mM)
Acetyl-L-tryptophanamide	5·3	.
Acetyl-D-tryptophanamide	.	2·7
Acetyl-L-tyrosinamide	30·5	.
Acetyl-D-tyrosinamide	.	12·0
Nicotinyl-L-tryptophanamide	2·7	.
Nicotinyl-D-tryptophanamide	.	1·4
Nicotinyl-L-tyrosinamide	15·0	.
Nicotinyl-D-tyrosinamide	.	6·2
Acetyl-L-tryptophan	.	17·5
Acetyl-D-tryptophan	.	4·8
L-Tryptophanamide	.	6·3
D-Tryptophanamide	.	3·2

Analogous results have been obtained by Niemann and his colleagues for α-chymotrypsin (see Table 6). With this enzyme D analogues of the synthetic substrates act as competitive inhibitors, K_i values for the D compounds being lower than the K_m values for the corresponding L compounds. This does not necessarily mean that the enzyme has a higher affinity for the D-isomer, since K_m may have a complex kinetic meaning and not be a direct measure of the affinity for substrate. However, with acetyltryptophan and tryptophanamide neither enantiomorph is hydrolysed by chymotrypsin, and the inhibitory action shows clearly that the affinity of the enzyme for the D-isomer is 2–3 times that for the L compound. These results emphasize the importance of distinguishing the two stages of enzyme action. If a molecule contains the correct combining groups, then in these cases either configuration will allow it to combine with the active centre, although the D arrangement allows it to bind more firmly. But only the L configuration brings about the mutual orientation of enzyme and substrate groups which produces activation and a completion of the catalytic process.

SOME OTHER HYDROLASES OF HIGH STEREOSPECIFICITY

A number of other hydrolytic enzymes have been shown to have a high stereospecificity. Arginase is specific for L(+)-arginine; D-arginine is not attacked and does not inhibit (Reisser, 1906; Edlbacher & Bonem, 1925). In this case other L-amino acids are inhibitors, but all D-amino acids are inactive (Hunter & Downs, 1945). The enzyme dihydro-orotase, which hydrolytically opens the ring of dihydro-orotate, giving ureidosuccinate, has no action on D-dihydro-orotate, and the rate with the DL compound is 88 % of that with the L compound alone (Lieberman & Kornberg, 1954). Bacterial ureidosuccinase splits off CO_2 and NH_3 from L-ureidosuccinate, but has no action on D-ureidosuccinate; here the rate with the DL compound is 94 % of the rate with L-ureido-succinate, so there can be very little affinity for the enantiomorph (Lieberman & Kornberg, 1955). Histidase is specific for L(−)-histi-dine, but this reaction is strongly inhibited by D-histidine (Edlbacher & Kraus, 1930; Edlbacher, Baur & Becker, 1940).

Fumarase is not strictly a hydrolytic enzyme but one in which ele-ments of water are added across a carbon–carbon double bond; it will be discussed here for completeness. It is absolutely specific, L-malate and fumarate being the only substrates. Dakin (1922) showed the asymmetric synthesis of L-malate from fumarate by this enzyme. Fumarase shows two kinds of stereospecificity, since in the first direc-tion it acts only on one of a pair of optical enantiomorphs, and in the other on only one of a pair of geometrical isomers. Although none of the compounds related to L-malate or fumarate are substrates, a number of them are strong competitive inhibitors, including D-malate and maleate, which are stereoisomers of the substrates (Massey, 1953). The specificity studies have been extended by the use of isotopes (Fisher, Frieden, McKee & Alberty, 1955). Fumarase was allowed to act on fumarate in 99·5 % D_2O, and the malate formed was isolated after thorough washing with H_2O. It contained 0·97 excess atom of deuterium/mol. On the other hand fumaric acid isolated from the digest had less than 10^{-4} atom/mol. of non-exchangeable deuterium. Thus the entering hydrogen atom is added in only one of the two possible positions on the –CH = group, and a hydrogen atom from the identical position is removed in the dehydration reaction.

The possibility that enzymes might under certain conditions dis-tinguish between identical atoms or groups was first discussed in an important theoretical contribution by Ogston (1948). If an enzyme has groups in the active centre which combine with any three of the sub-stituent groups in the substrate Cx_2yz, where C is a carbon atom, the

substrate can only be bound by the enzyme in one orientation, in spite of its being a symmetrical molecule. In that case it is perfectly feasible for the enzyme to activate only one particular x group, say for conversion into x', and an asymmetric compound $Cxx'yz$ will be produced. Stereospecificity of this type has been shown in a number of cases, for instance in the formation and utilization of citric acid. When this is formed enzymically from ^{14}C-labelled oxaloacetic acid

$$(\overset{*}{C}O_2H \cdot CH_2 \cdot CO \cdot CO_2H),$$

and the labelled citric acid converted by aconitase and *iso*citrate dehydrogenase into α-oxoglutarate, the ^{14}C is found to be entirely in the α-carboxyl group (Lorber, Utter, Rudney & Cook, 1950). This phenomenon, which can be detected only by making the molecule asymmetric by means of an isotopic label, has been called 'isotopic pseudo-asymmetry' (Wilcox, 1949). It is not completely unique to enzymes, since with optically-active non-biological catalysts a slightly optically active product of the type $Cxx'yz$ can be obtained from Cx_2yz (Schwartz & Carter, 1954). In these cases, however, there is merely a small difference of rate of reaction of the 'identical' groups, whereas in the enzymic cases the distinction between them is absolute.

Ogston (1958) has recently replied to some criticisms and misunderstandings of his earlier concept. He points out that the two forms of Cx_2yz can be distinguished only by means of an asymmetric agent, of which an enzyme is one. 'The essential feature is the dependence of the reactivity upon the mutual orientation of its parts. The phenomenon of discrimination is not ascribable to each part separately, but to the relationship between both.' The postulate of a three-point attachment between enzyme and substrate to explain such discrimination 'correctly expresses the uniqueness of any particular mutual orientation of an asymmetric molecule and one which contains two "identical" groups, upon which reactivity must depend'.

CONCLUSION

It is not true, as is sometimes stated, that all enzymes acting on asymmetric substrates are absolutely specific to one form or the other. In many cases the opposite enantiomorph is acted on at an appreciable rate, and, even when it is not activated, it may combine with the active centre with a considerable affinity, which may even exceed that for the substrate. Nevertheless, even in the less striking cases the degree of optical preference shown by enzymes is probably greater than in the best examples of asymmetric synthesis carried out with non-biological catalysts (Ritchie, 1947). With enzymes which show comparatively little

specificity for the chemical structure of the substrate, such as the ali-esterases, the degree of optical specificity found is surprisingly high.

Basically the facts reviewed here arise from the obvious asymmetry of the enzymes themselves, which are proteins made up entirely from L-amino acids. If this asymmetric composition is considered in conjunction with the requirement for a definite pattern of chemical groups in the substrate, which suggests enzyme–substrate combination at two or more points, it would perhaps be surprising to find any enzyme able to act on both components of a DL mixture at the same rate. So far as the author is aware, there is no example of an enzyme acting on an asymmetric substrate without some degree of stereospecificity.

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DISCUSSION OF PAPER BY WEBB

H. Gutfreund (*National Institute for Research in Dairying, Shinfield*): I should like to amplify Dr Webb's remarks about the specificity of chymotrypsin. Studies with a variety of techniques in the laboratories of Dr C. Niemann and Dr H. Neurath as well as in our own have

revealed some considerable detail of the individual steps of the reaction mechanism of this enzyme (for a summary of references see Gutfreund & Hammond, 1959).

We have been able to distinguish between three steps in the reaction between chymotrypsin (E) and substrate (AB):

$$\mathrm{E} + \mathrm{AB} \underset{}{\overset{k_1}{\rightleftharpoons}} \mathrm{E-AB} \overset{k_2}{\rightarrow} \mathrm{EA} + \mathrm{B} \overset{k_3}{\rightarrow} \mathrm{E} + \mathrm{A} + \mathrm{B}$$

The first step involves physical adsorption of the substrate on the specificity site, the second step is an imidazole-catalysed acylation of a serine hydroxyl group of the enzyme with concomitant liberation of the non-acyl moiety of the substrate and the third step is the hydrolysis of the enzyme acyl compound.

For specificity considerations we can isolate quantitatively the contribution of the initial adsorption to the binding energy. Chymotrypsin is usually regarded as specific for derivatives of aromatic amino acids, but it has been shown that a large variety of acyl derivatives of compounds with ring structures are hydrolysed by this enzyme. Since many different compounds with rings of widely different chemical properties (phenolic, aromatic, saturated) have approximately the same binding constants for chymotrypsin, one is justified in assuming that the specific binding is purely a physical adsorption of complementary structures.

A final answer to the questions about the absolute specificity of chymotrypsin for L-amino acid derivatives will have to await more detailed chemical information (Neurath & Hartley, 1959), but we should soon be in a position to build models of the interactions of the different parts of the specificity site and catalytic site of this enzyme with its substrate and thus explain its stereochemical specificity.

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STERIC FACTORS IN ENZYME ACTION: OXIDATION-REDUCTION ENZYMES

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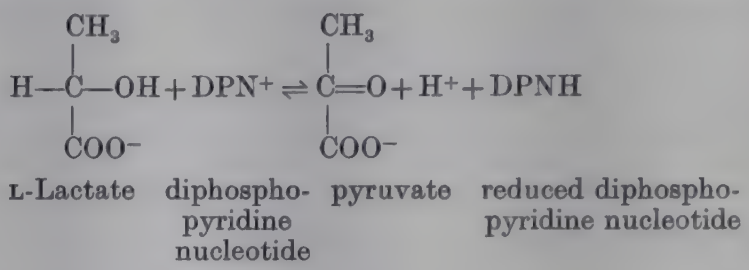
Many oxidizing enzymes, like other enzymes, are completely stereospecific. For example, the lactate dehydrogenase concerned in glycolysis in animal tissues is completely specific for L-lactate (Kubowitz & Ott, 1943).



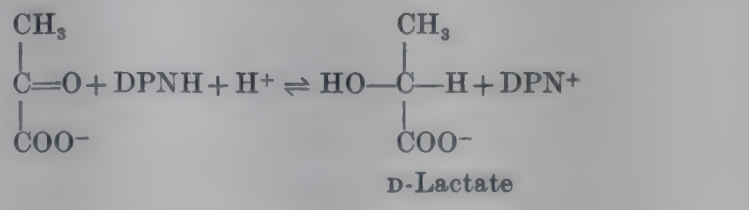
(1)

D-Lactate is not oxidized, and the reduction of pyruvate by DPNH catalysed by the enzyme yields only L-lactate.

In 1935, Krebs made the surprising discovery that animal tissues contain two different series of enzyme systems catalysing the oxidation of amino acids, one specific for L-amino acids, the other for the D-amino acids, until then considered metabolically inert (see Krebs, 1948). In recent years, the presence, sometimes in the same cell, of different enzymes specific for each of the two optical isomers has become commonplace. Moreover, a number of enzymes, called racemases or stereoisomerases, have been found which catalyse the interconversion of the two isomers.

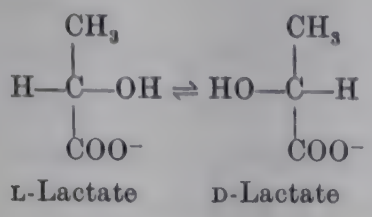
STEREOSPECIFIC DEHYDROGENASES AND RACEMASES

Lactate dehydrogenase. In addition to the well-known L-lactate dehydrogenase, D-specific enzymes have been reported in *Lactobacillus arabinosus* (Korkes, Del Campillo & Ochoa, 1950; Kaufman, Korkes & Del Campillo, 1951) and in liver and kidney cyclophorase preparations (Huennekens, Mahler & Nordmann, 1951). If the same cell contained L- and D-specific dehydrogenases, both reacting with DPN^+, and the products of their action were available to one another, reaction (1) followed by reaction (2):



(2)

would give the sum reaction (3):



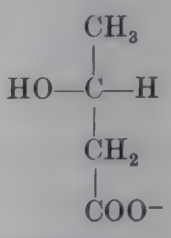
(3)

It is necessary to consider whether this is the mechanism whereby racemic mixtures are formed in the cell, or whether separate racemases are involved.

Kaufman *et al.* (1951) suggest that this is the mechanism operating in *L. arabinosus*, since the conversion of D- into L-lactate by enzyme preparations isolated from this organism occurred only in the presence of DPN^+. However, Kitahara, Obayashi & Kukui (1953) could find no evidence for the participation of lactate dehydrogenase in the enzyme catalysing reaction (3) which they isolated from *Lactobacillus plantarum*, a micro-organism which makes DL-lactate. Similarly, Ayengar & Roberts (1952) found that the rate of conversion of D-glutamate into L-glutamate by acetone-dried powders of *L. arabinosus* was much greater than the rate of reduction of α-oxoglutarate and NH_4^+ to L-glutamate, suggesting that glutamic dehydrogenase is not involved in this racemization.

Huennekens *et al.* (1951) and Mahler, Tomisek & Huennekens (1953) found that liver and kidney cyclophorase preparations are stereospecific for D-lactate, unless a racemase, prepared from the soluble fraction of the same tissue, is added. The racemase also reacts with other α-hydroxy acids, namely, malate, *iso*citrate and α-hydroxyglutarate. The oxidation of D-lactate by the cyclophorase preparations did not require added DPN^+, but it is possible that mitochondrial-bound DPN^+ is involved. Since the soluble fraction contains L-lactate dehydrogenase, it would appear possible that the mechanism shown in reactions (1)–(3) satisfactorily explains the racemase reaction. However, Huennekens *et al.* (1951) reject this explanation, since it would not accord with the fact that the same kidney or liver racemase preparations catalyse the oxidation of D-lactate by pigeon-breast-muscle cyclophorase preparations, which by themselves are completely stereospecific for the L form. Thus it appears more likely that a separate racemase is responsible for the interconversion of the two isomers. However, some connexion with the oxidizing activity of the mitochondria appears to be implied in the authors' belief 'that the racemase action does not occur in the absence of a functioning citric acid cycle as a continuous source of oxidative phosphorylation'. The mitochondrial D-lactate dehydrogenase has recently been isolated from rabbit-kidney mitochondria by Tubbs & Greville (1959).

β-Hydroxybutyrate and β-hydroxybutyryl-CoA dehydrogenases. β-Hydroxybutyrate dehydrogenase is stereospecific for the D(−) form (until recently referred to as *l*-β-hydroxybutyrate). Thus β-hydroxybutyrate formed by reduction of acetoacetate is the D(−) form. However, the enzyme which oxidizes and forms the coenzyme A (CoA) derivative of β-hydroxybutyrate is specific for the L(+) form.



D(−)-β-Hydroxybutyrate

These two enzymes, together with fatty acid activation enzyme and acetoacetyl-CoA deacylase, provide a mechanism for the conversion of L(+)-β-hydroxybutyrate into the D(−) form (Lehninger & Greville, 1953).

L(+)-β-Hydroxybutyrate + ATP + CoA ⇌ L(+)-β-hydroxybutyryl-CoA + AMP + PP (4)
L(+)-β-Hydroxybutyryl-CoA + DPN^+ ⇌ acetoacetyl-CoA + DPNH + H^+ (5)
Acetoacetyl-CoA + H_2O → acetoacetate + CoA (6)
Acetoacetate + DPNH + H^+ ⇌ D(−)-β-hydroxybutyrate + DPN^+ (7)

Sum reaction: L(+)-β-Hydroxybutyrate + ATP + H_2O → D(−)-β-hydroxybutyrate + AMP + PP (8)

The sum reaction is a Walden inversion (not a racemization, as stated by Lehninger & Greville, 1953) of L(+)-β-hydroxybutyrate into the D(−) form, coupled with the hydrolysis of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and inorganic pyrophosphate (PP).

In the presence of acetoacetate-activating enzyme, L(+)-β-hydroxybutyryl-CoA can be synthesized from D(+)-β-hydroxybutyrate according to reactions (9)–(11):

D(−)-β-Hydroxybutyrate + DPN^+ ⇌ acetoacetate + DPNH + H^+ (9)
Acetoacetate + ATP + CoA ⇌ acetoacetyl-CoA + AMP + PP (10)
Acetoacetyl-CoA + DPNH + H^+ ⇌ L(+)-β-hydroxybutyryl-CoA + DPN^+ (11)

Sum reaction: D(−)-β-Hydroxybutyrate + ATP + CoA ⇌ L(+)-β-hydroxybutyryl-CoA + AMP + PP (12)

Although both Walden inversions must proceed in the presence of the appropriate enzymes, the quantitative importance of these reactions *in vivo* is not known. There is another enzyme, a true racemase, present in ox and rat liver, which catalyses the racemization of β-hydroxybutyryl-CoA (Stern, Del Campillo & Lehninger, 1955). According to these authors, this enzyme appears to be independent of the presence of DPN^+, and preparations of ox and rat liver possessing racemase activity were devoid of DPN^+-linked dehydrogenases acting on either

D(−)- or L(+)-β-hydroxybutyrate, and of enzymes bringing about racemization of these isomers. However, Wakil (1955) has reported an additional enzymic mechanism present in beef-liver mitochondria capable of bringing about the racemization of β-hydroxybutyryl-CoA which is dependent on the operation of L(+)- and D(−)-specific dehydrogenases, following a course similar to that described in reactions (1)–(3).

L(+)-β-Hydroxybutyryl-CoA + DPN^+ ⇌ acetoacetyl-CoA + DPNH + H^+ (5)

Acetoacetyl-CoA + DPNH + H^+ ⇌ D(−)-β-hydroxybutyryl-CoA + DPN^+ (13)

Sum reaction: L(+)-β-Hydroxybutyryl-CoA ⇌ D(−)-β-hydroxybutyryl-CoA (14)

AMINO ACID OXIDASES

The function of the considerable quantities of D-amino acid oxidase in vertebrate kidney and liver is still not known. It cannot function as part of a racemase system, since the reaction which it catalyses:

$$\text{D-R.CH(NH}_2\text{).CO}_2\text{H} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{R.CO.CO}_2\text{H} + \text{H}_2\text{O}_2 + \text{NH}_3 \quad (15)$$

is essentially irreversible. A combination of reaction (15) with a DPN^+-linked L-amino acid dehydrogenase could lead to a Walden inversion, since the sum of reactions (15) and (16) is reaction (17):

$$\text{R.CO.CO}_2\text{H} + \text{DPNH} + \text{H}^+ + \text{NH}_3 \rightleftharpoons \text{L-R.CH(NH}_2\text{).CO}_2\text{H} + \text{DPN}^+ + \text{H}_2\text{O} \quad (16)$$

$$\text{D-R.CH(NH}_2\text{).CO}_2\text{H} + \text{DPNH} + \text{H}^+ + \text{O}_2 \rightarrow \text{L-R.CH(NH}_2\text{).CO}_2\text{H} + \text{DPN}^+ + \text{H}_2\text{O}_2 \quad (17)$$

However, the most active pyridine nucleotide-linked L-amino acid dehydrogenase is glutamate dehydrogenase, and D-glutamate is almost completely unattacked by D-amino acid oxidase (Krebs, 1948). Nevertheless, it is probable that a mechanism of this type operated in the inversion of leucine found by Ratner, Schoenheimer & Rittenberg (1940). D-Leucine labelled with both ^{15}N and ^2H was fed to rats. The L-leucine isolated from the tissues contained little ^{15}N, but all the ^2H which was not in the α-position was retained [see Neuberger (1948) for a further discussion of these experiments].

The hypothesis that D-amino acid oxidase might play a part in the synthesis of L-amino acids by resolving DL-amino acids formed by an asymmetric synthesis was made improbable by the isotope experiments of Shemin & Rittenberg (1943). After administration of ^{15}N-labelled DL-tyrosine and DL-glutamate, no dilution in the isotope content of the D-amino acid excreted in the urine was found.

One physiological function of D-amino acid oxidase is presumably to oxidize D-amino acids introduced with the food. It still remains a puzzle, however, why such a high activity is necessary to deal with the relatively small amounts of D-amino acids found in Nature.

STEREOSPECIFICITY OF HYDROGEN TRANSFER IN REACTIONS OF PYRIDINE NUCLEOTIDE DEHYDROGENASES

A large number of dehydrogenases are known which catalyse reaction (18) (Fig. 1), where R represents the ribose–pyrophosphate–adenosine moiety of DPN^+ or the ribose–pyrophosphate–2′-phosphoadenosine moiety of triphosphopyridine nucleotide (TPN^+).

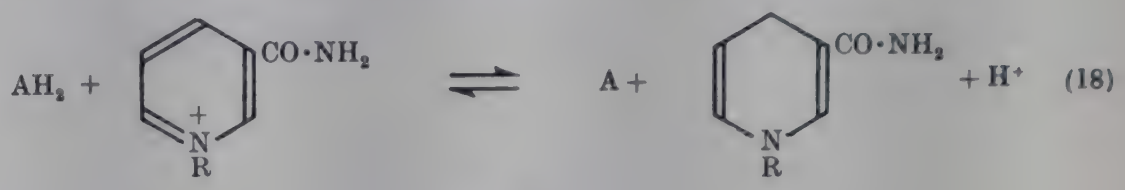


Fig. 1.

Most of these dehydrogenases fall into two groups, called alcohol and aldehyde dehydrogenases (cf. Van Eys, San Pietro & Kaplan, 1958). The largest group, the alcohol dehydrogenases, oxidize primary alcohols to aldehydes (e.g. alcohol dehydrogenase), secondary alcohols to ketones (e.g. lactate dehydrogenase), primary amines to ketones and ammonia (e.g. glutamate dehydrogenase), and hemiacetals to lactones (e.g. glucose 6-phosphate dehydrogenase). The aldehyde dehydrogenases oxidize aldehydes to carboxylic acids. There is also one enzyme (dihydro-orotate dehydrogenase) which catalyses the removal of hydrogen atoms from adjacent carbon atoms, with the formation of a C=C double bond (reaction 25).

Table 1. *Hydrogen transfer with alcohol dehydrogenase*

These results are compiled from Fisher, Conn, Vennesland & Westheimer (1953) and Loewus, Ofner, Fisher, Westheimer & Vennesland (1953). ADH, alcohol dehydrogenase; LDH, lactic dehydrogenase.

					Atom of ^2H/mol. in		
Expt.	Reductant	Oxidant	Medium	Enzyme	Acetaldehyde	DPNH	DPN^+
1	$Na_2S_2O_4$	DPN^+	^2H_2O	None	—	1·01	—
2	$CH_3 \cdot CH_2 \cdot OH$	DPN^+	^2H_2O	ADH	—	0	—
3	$CH_3 \cdot C^2H_2 \cdot OH$	DPN^+	H_2O	ADH	1·00	1·00	—
4	DPN^2H*	$CH_3 \cdot CHO$	H_2O	ADH	—	—	0
5	DPN^2H†	$CH_3 \cdot CHO$	H_2O	ADH	—	—	0·44
					Lactate		
6	DPN^2H*	$CH_3 \cdot CO \cdot CO_2H$	H_2O	LDH	0·97	—	—
7	DPN^2H†	$CH_3 \cdot CO \cdot CO_2H$	H_2O	LDH	0·58	—	—

* Prepared with $CH_3 \cdot C^2H_2 \cdot OH$ and alcohol dehydrogenase (cf. Expt. 3).
† Prepared with $Na_2S_2O_4$ (cf. Expt. 1).

Vennesland and her co-workers have studied the stereospecificity of various dehydrogenases, by using substrates labelled with deuterium (^2H) (reviewed by Vennesland, 1955, 1956, 1958). The first experiments

with alcohol and lactate dehydrogenases are summarized in Table 1. Expt. 1 shows that reduction of DPN^+ with $Na_2S_2O_4$ in the presence of ^2H_2O yields DPN^2H, containing 1 atom of deuterium. This deuterium atom was not lost when the compound was dissolved in H_2O, showing that it is non-exchangeable. Expt. 2 shows that none of the hydrogen in DPNH formed by reduction of DPN by ethanol in the presence of alcohol dehydrogenase is derived from water. Expt. 3 with dideutero-ethanol ($CH_3 \cdot C^2H_2 \cdot OH$), DPN^+ and alcohol dehydrogenase showed that both products, DPNH and $CH_3 \cdot CHO$, contained one atom of ^2H per molecule. When this DPNH was oxidized by unlabelled $CH_3 \cdot CHO$ in the presence of alcohol dehydrogenase (Expt. 4), or by pyruvate in the presence of lactic dehydrogenase (Expt. 6), all the deuterium was removed from the coenzyme, and found in the alcohol (see below) or in the lactate (Table 1) respectively.

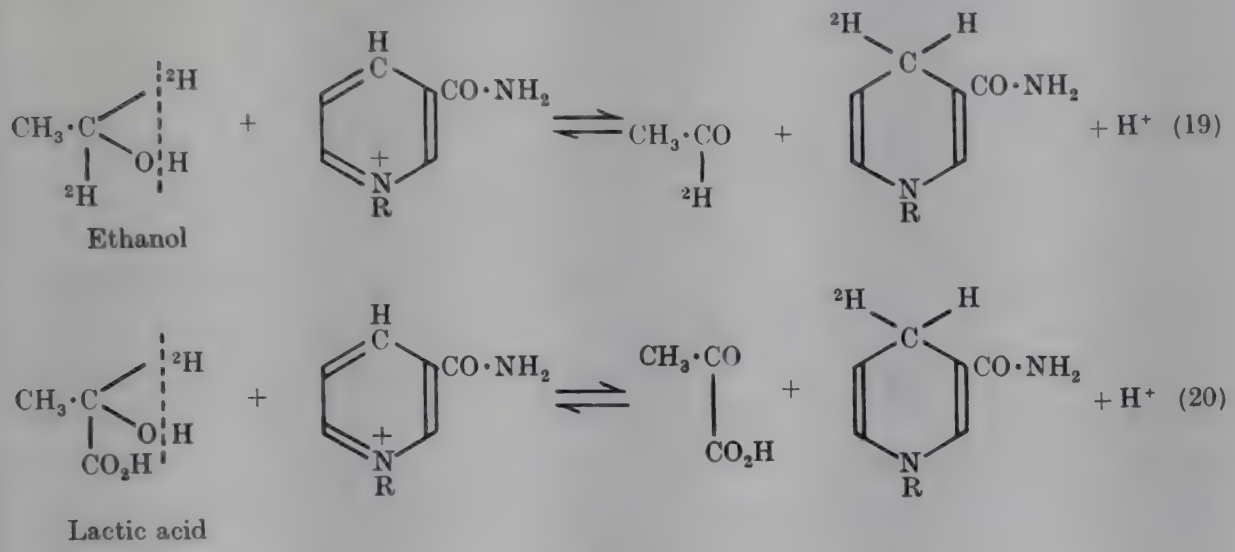


Fig. 2.

The reactions may be written as in Fig. 2. From this experiment, certainly one of the most interesting ever performed in the study of the mechanism of enzyme action, a number of conclusions of importance for the discussion of the stereochemistry of this class of enzymes can be drawn:

(*a*) The orientation of the substrate with respect to the pyridine nucleotide is always the same. This is shown by the fact that the hydrogen atom directly attached to the carbon atom in ethanol is transferred to C-4 of the coenzyme, while the hydrogen atom on the hydroxyl group is transferred to the nitrogen atom, and is then (at usual pH values) dissociated as a H^+ ion. If the orientation had been reversed, some of the deuterium would have been lost to the solution as a $^2H^+$ ion.

(*b*) The enzyme reaction is specific for one face of the pyridine ring, since it reacts with only one of the two hydrogen atoms attached to C-4

in the pyridine ring of DPNH. If the enzyme could not choose between these chemically indistinguishable hydrogen atoms, the back reaction between DPN^{2}H and $CH_3 \cdot CHO$ would have left one half of the ^{2}H in the DPN$^+$. Thus the dehydrogenase catalyses the transfer of hydrogen atoms to, and removal from, one side of the pyridine ring. On the other hand, non-enzymic reduction of DPN$^+$ with $Na_2S_2O_4$ (Yarmolinsky & Colowick, 1956) in ^2H_2O yields DPN^{2}H in which the ^{2}H is distributed between the two positions. This is shown by the fact that oxidation of this product with acetaldehyde in the presence of alcohol dehydrogenase (Table 1, Expt. 5) or with pyruvate in the presence of lactate dehydrogenase (Expt. 7) removes only about 57 % of the ^{2}H [72 % in the experiments of Pullman, San Pietro & Colowick (1954)].

Table 2. *Hydrogen transfer with glucose dehydrogenase*

These results are from Levy, Loewus & Vennesland (1956).

Expt.	Reductant	Oxidant	Atom of ^{2}H/mol. in Lactate	Atom of ^{2}H/mol. in DPN$^+$
1	[1-^{2}H]Glucose	DPN$^+$	0·007	0·92
2	Glucose	[4-^{2}H]DPN$^+$	0·133	0

(*c*) Alcohol dehydrogenase and lactate dehydrogenase react with the same face of the pyridine ring, since the ^{2}H transferred to it from dideuteroethanol by alcohol dehydrogenase was removed by lactate dehydrogenase. Further studies showed that some dehydrogenases acted on the same face as alcohol and lactate dehydrogenases (called the α or A face), while other dehydrogenases reacted on the other (β or B) face.

The experimental method used by Vennesland to determine the stereospecificity of the different enzymes is to introduce ^{2}H into DPNH by reaction of DPN$^+$ with ^{2}H-labelled substrate, and then to allow the DPN^{2}H to react with pyruvate and lactate dehydrogenase as described above. The isolated DPN$^+$ and lactate (as phenacyl derivative) are then analysed for deuterium. If ^{2}H is retained in the re-formed DPN$^+$, the dehydrogenase is specific for the B face. If the ^{2}H is removed to the lactate, it is specific for the A face. An alternative method is to introduce deuterium at the C-4 of DPN$^+$ by treating the latter with concentrated cyanide and alkali in ^2H_2O (San Pietro, 1955). The [^{2}H]DPN$^+$ is then reduced with unlabelled substrate to give DPN^{2}H containing deuterium on the opposite face to that used by the enzyme.

Results obtained by the two methods for glucose dehydrogenase are shown in Table 2, taken from Levy, Loewus & Vennesland (1956). In Expt. 1, unlabelled DPN$^+$ was reduced with [1-^{2}H]glucose, and in Expt. 2 [4-^{2}H]DPN$^+$ was reduced by unlabelled glucose. In both cases

the DPN^{2}H was isolated and oxidized with pyruvate in the presence of lactate dehydrogenase, and the lactate and DPN$^+$ were analysed for ^{2}H. The results show clearly that glucose dehydrogenase has the opposite stereospecificity to that of lactate dehydrogenase, i.e. that it reacts with the B face.

Table 3. *Steric specificity of pyridine nucleotide dehydrogenases*

These results are compiled from Vennesland (1958).

	DPN$^+$ dehydrogenases
A-specific	Ethanol (yeast, *Pseudomonas*, liver, wheat germ)
	Acetaldehyde (liver)
	L-Lactate (heart)
	L-Malate (heart, wheat germ)
	D-Glycerate (spinach)
	Dihydro-orotate (*Zymobacterium oroticium*)
B-specific	α-Glycerophosphate (skeletal muscle)
	3-Phosphoglyceraldehyde (yeast, skeletal muscle)
	L-Glutamate (liver)
	D-Glucose (liver)
	β-Hydroxy steroid (*Pseudomonas*)
	L-β-Hydroxybutyryl-CoA (heart)
	TPN$^+$ dehydrogenases
A-specific	*iso*Citrate (heart)
B-specific	L-Glutamate (liver)
	Glucose 6-phosphate (yeast)

A third method used by Levy & Vennesland (1957) which is particularly suitable when the equilibrium of the reaction lies to the side of DPN$^+$ is to prepare the two DPN^{2}H's, one with the deuterium on the A face (prepared with labelled ethanol and unlabelled DPN$^+$), and the second with deuterium on the B face (prepared with unlabelled ethanol and labelled DPN$^+$). Reaction of these DPN^{2}H's with α-oxoglutarate

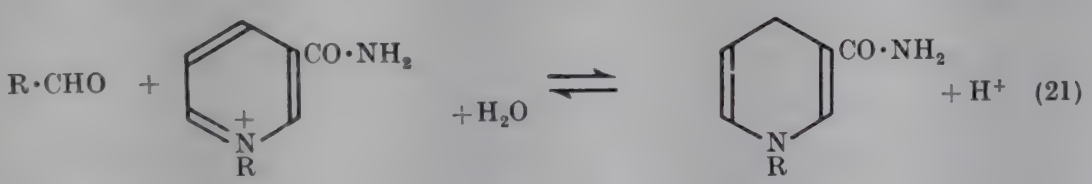


Fig. 3.

and ammonia in the presence of glutamate dehydrogenase showed that the A-labelled DPN^{2}H retained its deuterium on oxidation while the deuterium in the B-labelled DPN^{2}H was transferred to glutamate. Thus glutamate dehydrogenase is B-specific.

Table 3, compiled from Vennesland (1958), lists the stereospecificity of all the DPN$^+$- and TPN$^+$-reacting dehydrogenases which have been examined. Most of these enzymes are of the alcohol type, but there is also the special case of dihydro-orotate dehydrogenase, as well as two enzymes of the aldehyde type, namely acetaldehyde and 3-phosphoglyceraldehyde dehydrogenase, which catalyse reactions (21) (Fig. 3).

In this case, also, the hydrogen atom bound to the carbon atom in the aldehyde groups is transferred directly to C-4 of the pyridine ring. With phosphoglyceraldehyde dehydrogenase, however, there is also an appreciable exchange of hydrogen atoms with water. This was shown by reducing the DPN^+ by the substrate in the presence of the enzyme in a medium of about 100% ^2H_2O. When the reduction was complete, the enzyme was inactivated and the ^2H_2O removed. The DPNH was dissolved in H_2O and oxidized by pyruvate in the presence of lactic dehydrogenase, and the deuterium content of the DPN^+ and lactate measured. 0·06 Atom of ^2H/molecule was found in the DPN^+ and 0·04–0·13 in the lactate. A similar experiment with glucose and glucose dehydrogenase revealed only 0·01 atom/molecule in both the DPN^+ and the lactate.

It is of interest that alcohol dehydrogenases from different sources all react with the same side of the pyridine ring, although there are considerable differences in other properties.

Glutamate dehydrogenase, which operates almost equally well with both DPN^+ and TPN^+, reacts in both cases with the B side of the pyridine ring. The stereospecificity for the TPNH was shown by preparing TPN^2H by reduction of [4-^{2}H]TPN^+ (prepared chemically) with *iso*citrate in the presence of *iso*citrate dehydrogenase. The isolated TPN^2H was in one experiment allowed to react with α-oxoglutarate ($+NH_4^+$) in the presence of glutamate dehydrogenase, and in another experiment was converted into DPN^2H by enzymic hydrolysis of the phosphate group. The configuration of the DPN^2H was established to be of the B type by finding that the deuterium was retained in the pyridine nucleotide, when it reacted with pyruvate in the presence of lactate dehydrogenase. Thus *iso*citrate dehydrogenase must be A-specific (cf. Englard & Colowick, 1957) and, since glutamate dehydrogenase transferred the deuterium from the TPNH to glutamate, the latter enzyme must be B-specific (Vennesland, 1958).

It may be significant that glyceraldehyde 3-phosphate dehydrogenase, on the one hand, and lactate and alcohol dehydrogenases, on the other, have opposite stereospecificities, since the DPNH made by the former enzyme is oxidized in glycolysis and fermentation respectively by the latter enzymes. Cori, Velick & Cori (1950), Nygaard & Rutter (1956) and Astrachan, Colowick & Kaplan (1957) have shown that DPNH bound to glyceraldehyde phosphate dehydrogenase reacts with pyruvate in the presence of lactate dehydrogenase, as rapidly as or more rapidly than free DPNH. It is perhaps easier to visualize a mechanism in which one side of the pyridine ring receives a hydrogen atom from phosphoglyceraldehyde, while the hydrogen atom on the other side of the ring is transferred to pyruvate. However, Astrachan *et al* (1957) have shown that α-oxoglutarate ($+NH_4^+$) in the presence of glutamate

dehydrogenase, which has B specificity, also readily reacts with DPNH bound to active phosphoglyceraldehyde dehydrogenase.

The stereospecificity of enzymes which catalyse the oxidation of DPNH by other respiratory carriers has also been studied. Drysdale & Cohn (1956) showed that the DPNH oxidase system of liver mitochondria and the DPNH–cytochrome *c* reductase of rat liver and of pig-heart muscle had B specificity. This is also the case with pyridine nucleotide transhydrogenase of *Pseudomonas*, which catalyses reaction 22 (San Pietro, Kaplan & Colowick, 1955),

$$\text{TPNH} + \text{DPN}^+ \rightarrow \text{TPN}^+ + \text{DPNH} \qquad (22)$$

and with the TPNH-glutathione reductase of *Escherichia coli* (Vennesland, 1958).

Pig-heart diaphorase does not have a high degree of stereospecificity for DPNH, preferring the B side to the extent of only 69 % (Weber,

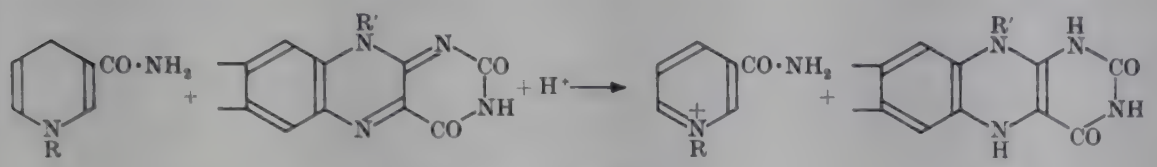


Fig. 4.

Kaplan, San Pietro & Stolzenbach, 1957). Vennesland (1956) has reported also the same degree of preference (65 %) for the B side for the oxidation of DPNH by methylene blue catalysed by a xanthine-oxidase preparation. This would appear to provide some support for Morell's (1952) contention that the diaphorase activity of xanthine-oxidase preparations is due to a contaminating flavoprotein rather than to xanthine oxidase itself.

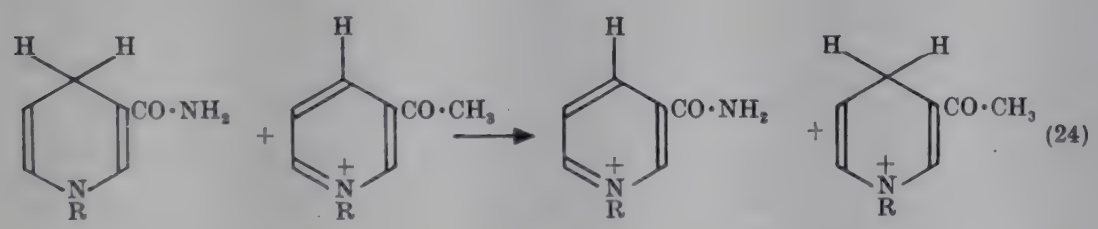


Fig. 5.

In the oxidation of DPNH by flavin [reaction (23), Fig. 4] it is of interest to know whether one of the two extra hydrogen atoms in the reduced flavin is derived from the DPNH, and if so which one. It is, however, not feasible to isolate the reduced flavin and analyse it for deuterium, since the extra hydrogen atoms on reduced flavin exchange rapidly with the medium.

Weber *et al.* (1957) have studied hydrogen transfer in reaction 24 (Fig. 5), which is catalysed by flavoproteins, containing flavinadenine

dinucleotide, such as diaphorase and cytochrome reductase (Weber & Kaplan, 1957). They found that all the extra hydrogen in the reduced pyridine nucleotide analogue was derived from the medium (^2H_2O), showing that exchange had taken place. This also appears to be the case with dihydro-orate dehydrogenase, which catalyses reaction (25) (Fig. 6). This enzyme, like others catalysing the removal or addition of two hydrogen atoms from adjacent carbon atoms, is a flavoprotein. Although it is completely A-specific, as shown by the fact that all the deuterium was removed from A-DPN^{2}H but none from B-DPN^{2}H, no deuterium ($< 1\%$) was found in the dihydro-orotate (Graves & Vennesland, 1957; Vennesland, 1958).

Drysdale & Cohn (1956) have studied an interesting exchange reaction between DPNH and ^2H_2O which is catalysed by liver mito-

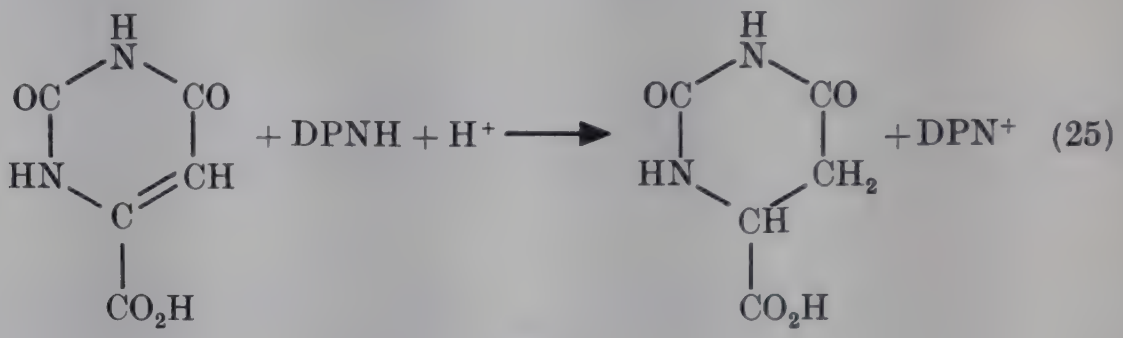


Fig. 6.

chondria in the presence of cyanide (to prevent oxidation of DPNH). This exchange reaction, which like the oxidation of DPNH by liver mitochondria is B-specific, is possibly catalysed by reaction (23) proceeding in reverse, under conditions favourable to oxidative phosphorylation. The deuterium would enter the reduced flavin by exchange with ^2H_2O, and would then be transferred to the pyridine nucleotide if reaction (23) is reversible. However, it is also possible that the exchange occurs between DPN ~ I (a hypothetical intermediate in oxidative phosphorylation, see Purvis, 1958) which might be the first product of the oxidation of DPNH by flavoprotein in mitochondria. If DPN ~ I is an addition compound of DPN^+ similar in type to that formed with cyanide, exchange would be expected (San Pietro, 1955). Thus the DPN^+ formed by transfer of I in the following reaction of the oxidative phosphorylation sequence would contain deuterium, and this would go to the B side of the pyridine ring if the dehydrogenases catalysing the reduction of the DPN^+ by endogenous substrates were A-specific.

Mechanism of action of alcohol-type dehydrogenases. Loewus, Westheimer & Vennesland (1953) showed that alcohol dehydrogenase has steric specificity for one of the hydrogen atoms in the CH_2 group of the

ethanol. [1-^{2}H]Ethanol was synthesized in the two ways shown in reactions (26) and (27), running from left to right:

$$CH_3.\overset{\overset{^2H}{|}}{C}O + DPNH + H^+ \rightleftharpoons CH_3.\overset{\overset{^2H}{|}}{\underset{\underset{H}{|}}{C}}.OH + DPN^+ \qquad (26)$$

$$CH_3.\overset{\overset{H}{|}}{C}O + DPN^2H + H^+ \rightleftharpoons CH_3.\overset{\overset{H}{|}}{\underset{\underset{^2H}{|}}{C}}.OH + DPN^+ \qquad (27)$$

The two [1-^{2}H]ethanols formed were found to be enantiomorphs. When the ethanol formed by reaction (26) was oxidized by DPN^+ it yielded acetaldehyde containing deuterium and unlabelled DPN^+. When the ethanol formed by reaction (27) was oxidized by DPN^+, it formed DPN^2H and unlabelled acetaldehyde. By the operation of reaction (26) from right to left, followed by reaction (27) from left to right, it was possible to

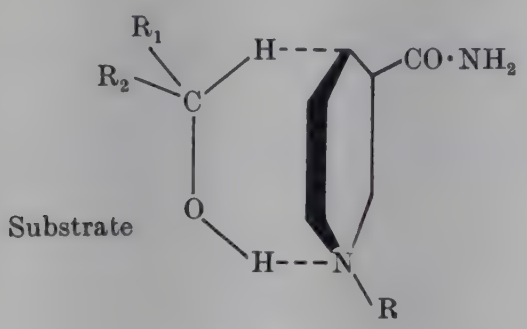


Fig. 7.

perform a Walden inversion. The [1-^{2}H]ethanol produced by reaction (26) has been isolated and found to be optically active (Vennesland, 1955).

Vennesland (1956) has pointed out that it is not necessary to assume that a substrate must be bound to the enzyme at three different points in order to explain steric specificity. What is required is a particular orientation of the reacting molecules, and steric hindrances may be just as important as attractive forces for the determination of this orientation.

Dixon & Webb (1958) have proposed the following mechanism of action of a typical dehydrogenase. They assume that the reactants are combined side by side on the enzyme surface, with their active groups close together (Fig. 7). The nicotinamide ring, seen on edge, is assumed to present one face to the substrate and to be prevented from turning by the amide group. If the bonds are drawn with the correct lengths, the reacting H atoms are found to be close to the positions which they occupy in $DPNH_2^+$. Dixon & Webb picture the activated complex as a resonance-stabilized structure in which the hydrogen atoms are shared

between substrate and DPN^+, and which may break down in either direction, into substrate and DPN^+, or into oxidized substrate and DPNH.

Van Eys *et al.* (1958) have proposed a rather different mechanism, in which the alcohol reacts with the DPN^+, bound to the enzyme, without itself being bound to the enzyme. This mechanism is based on the observation that in many cases the presence of a dehydrogenase promotes a much more favourable reaction between nucleophilic substances and pyridine nucleotides, compared with the extent of the reaction in the absence of the enzyme (Kaplan & Ciotti, 1954; Kaplan, 1955; Tereyama & Vestling, 1956; Pfleiderer, Jeckel & Wieland, 1956; Van Eys, Stolzenbach, Sherwood & Kaplan, 1958). These nucleophilic substances are of two types: (*a*) ions, such as HS^-, HSO_3^- and CN^-, which are bound with the stoicheiometry of 1 mole per 1 mole of DPN^+ bound to

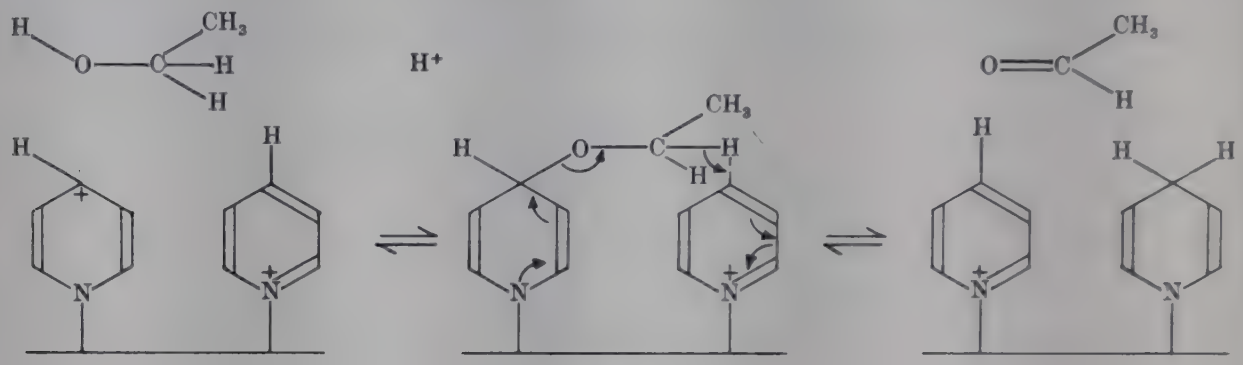


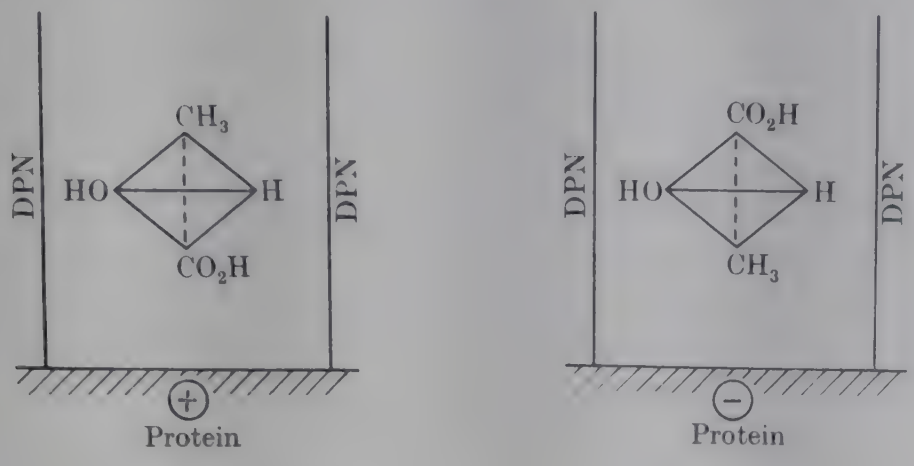
Fig. 8.

the enzyme, and (*b*) mercaptans, closely related to the substrate in structure, which are bound with the stoicheiometry of 1 mole per 2 moles of bound DPN^+ (except for yeast alcohol dehydrogenase, in which 1 complex is formed per 4 moles of DPN^+). All these substances are competitive inhibitors.

According to the Van Eys mechanism two molecules of DPN^+ are combined close to one another on the enzyme. The substrate binds through the hydroxyl (or amino) group to C-4 of one of the DPN^+ molecules with liberation of a H^+ ion (cf. Burton & Kaplan, 1954). By means of a simple electron shift from the nitrogen atom of one DPN molecule to the nitrogen of a second molecule, the second molecule of DPN^+ accepts a hydride ion from the substrate, and becomes reduced, while the first DPN^+ molecule and the substrate are regenerated. Both DPN^+ molecules are equally effective in accepting substrate or hydrogen (Fig. 8).

The role of the protein is to impart specificity to the dehydrogenase action by determining the relative distance and spatial arrangement of the DPN^+ molecules. A specific geometric configuration of the DPN^+

pair will also result in stereospecificity, both with regard to substrate and pyridine nucleotide. Charged groups on the protein surface could also determine the stereospecificity for the substrate (Fig. 9).



D-Lactate dehydrogenase

L-Lactate dehydrogenase

Fig. 9.

SUCCINATE DEHYDROGENASE

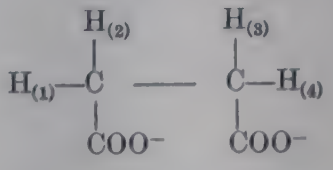
Succinate dehydrogenase is a flavoprotein, the prosthetic group of which is presumably reduced during operation of the enzyme:

$$\begin{array}{l} CH_2.COO^- \\ | \\ CH_2.COO^- \end{array} + fp \rightleftharpoons \begin{array}{l} CH.COO^- \\ \| \\ CH.COO^- \end{array} + fpH_2 \rightleftharpoons fpH^- + H^+$$

fp and fpH_2 represent the oxidized and reduced flavoprotein respectively. Since reduced flavoproteins rapidly exchange their extra hydrogen atoms with water, it would be expected that succinate dehydrogenase would catalyse an anaerobic exchange reaction between the hydrogen atoms of water and those in the methylene groups of succinate. Such an exchange has been demonstrated by Weinmann, Morehouse & Winzler (1947) and Englard & Colowick (1956). The latter authors showed that the reaction was greatly accelerated by the addition of fumarate.

Englard & Colowick found that when [^{2}H]succinate, prepared by the anaerobic exchange reaction between succinate and ^2H_2O catalysed by succinate dehydrogenase, was oxidized aerobically by the same enzyme preparation the fumarate formed retained about one-half of the deuterium, showing that succinate dehydrogenase, unlike the pyridine nucleotide dehydrogenases, does not distinguish between the hydrogen atoms attached to either carbon atom. They point out that this may be a reflexion of the symmetrical nature of the succinate molecule, and does not necessarily indicate that succinate dehydrogenase operates in

a fundamentally different way from the pyridine nucleotide dehydrogenases. Thus observation of a three-dimensional model of succinate (I) reveals that hydrogen atoms $H_{(1)}$ and $H_{(3)}$ are stereochemically indis-



tinguishable from one another, as also are $H_{(2)}$ and $H_{(4)}$. If the enzyme removes the pair $H_{(1)}$, $H_{(4)}$, this is stereochemically indistinguishable from removal of the pair $H_{(2)}$, $H_{(3)}$ and there is no enzyme or other device which could make the distinction. This argument is independent of the configuration of the carboxyl groups.

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DISCUSSION OF PAPER BY SLATER

Dr W. Klyne (*Postgraduate Medical School, London, W.* 12) drew attention to the important work on microbiological oxidations and reductions of simple decalones and hexahydroindanones by Prelog and his colleagues in Zürich [*Helv. chim. acta* (1956), **39**, 748; (1958), **41**, 1416, 1424, 1428, 2362, 2379, 2396; (1959), **42**, 736, 1239]. The generalized picture of these reactions, in which protein enzyme, nicotinamide coenzyme and substrate are represented in parallel planes [cf. *Ciba Foundation Study Group* (1959), no. 2, pp. 88–90. London: J. and A. Churchill Ltd.], constitutes a valuable extension of Ogston's concept of three-point attachment.

Dr H. R. Levy: It is of interest to emphasize that the stereospecificity with respect to the A or B side of the pyridine nucleotide is essentially another example of the ability of enzymes to distinguish between two apparently identical groups attached to what Schwartz & Carter (1954) have termed a *meso* carbon atom. Dr Webb mentioned fumarase as another example (Fisher, Frieden, McKee & Alberty, 1955). Other enzymes which have been found capable of activating one of two apparently identical hydrogen atoms on a *meso* carbon atom *Cxxyz* include aspartase (Englard, 1958; Krasna, 1958), aconitase (Englard & Colowick, 1957), phosphoglucose isomerase and phosphomannose isomerase (Topper, 1957), triose phosphate isomerase and aldolase (Rose &

DISCUSSION

Rieder, 1955; Bloom & Topper, 1956), and glycollic acid oxidase (Rose, 1958). Aldolase and triose phosphate isomerase have opposite stereospecificities for the hydrogen atoms on the carbinol carbon atom of dihydroxyacetone phosphate (Rose & Rieder, 1955; Bloom & Topper, 1956; Rose, 1958); phosphoglucose isomerase and phosphomannose isomerase activate different hydrogen atoms on C-1 of fructose 1-phosphate (Topper, 1957); these are further examples of pairs of enzymes with opposite stereospecificities analogous to those with opposite specificities for the two hydrogen atoms in DPNH. It would, of course, be of great interest to know what factors dictate this stereospecificity. Our examination of this question in DPN-linked dehydrogenases has not yet revealed any clear-cut chemical basis for differences in stereospecificity (Levy & Vennesland, 1957).

A point which is of considerable interest in this connexion has arisen from some recent experiments in which stereospecificity with respect to the hydrogen atoms on DPNH has been determined for enzymes oxidizing diastereoisomeric substrates. Jarabak, in Talalay's laboratory, has shown that the oxidation of androsterone by 3α-hydroxy steroid dehydrogenase and of *epi*androsterone by 3β-hydroxy steroid dehydrogenase both proceed with the same (side B) stereospecificity (Talalay & Levy, 1959). Similarly Kaplan (1959) has shown that D-lactic acid dehydrogenase and L-lactic acid dehydrogenase of *L. arabinosus* have the same (side A) stereospecificity. Thus no relationship appears to exist between optical configuration of the substrate and optical configuration of the reacting hydrogen atom of the DPN.

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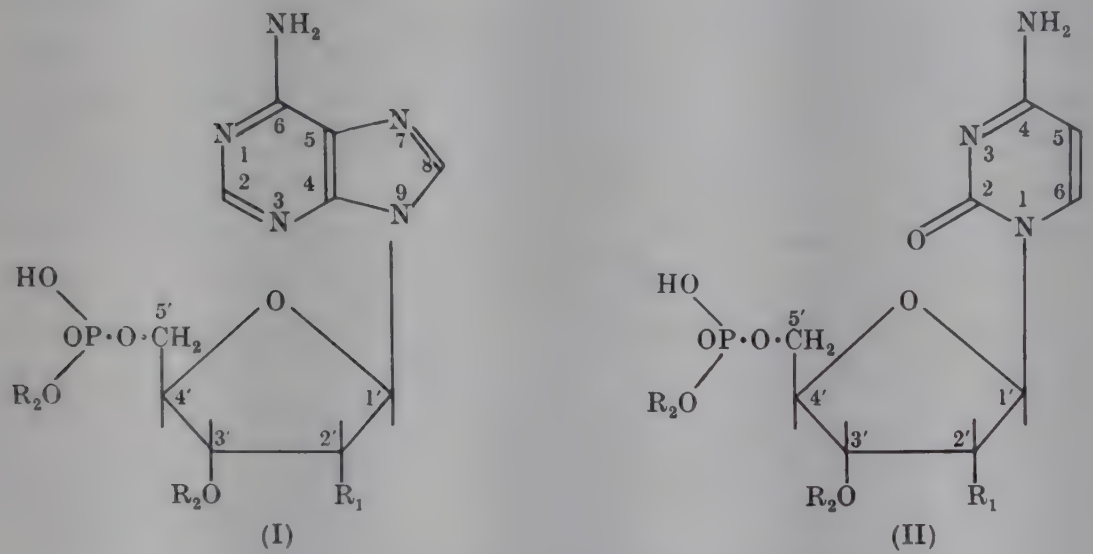
STEREOCHEMICAL SPECIFICITIES OF SOME ENZYMES OF NUCLEOTIDE METABOLISM

By G. R. BARKER

Department of Chemistry, The University, Manchester

A considerable number of enzymic reactions leading to the synthesis or breakdown of nucleotides and polynucleotides have now been carried out in cell-free systems, and it is the purpose of this review to consider the steric course of some of them. Few variations in the general structural patterns present in nucleotides and polynucleotides have been found, and it is therefore to be expected that the enzymes concerned in the formation of these structures will exhibit a high degree of specificity. In considering the contribution of stereochemical effects to these specificities, it is necessary to take account not only of selective forma-



R_1 = H or OH; R_2 = H or PO_3H_2 or a nucleotide etc.

tion of a given stereoisomer, but also to bear in mind that steric factors may play an important part in determining the general course of a reaction involving, for example, the production of one or another position isomer.

Examination of the structures of a typical purine (I) and pyrimidine (II) nucleotide reveals a number of ways in which enzymic specificity is concerned in their synthesis.

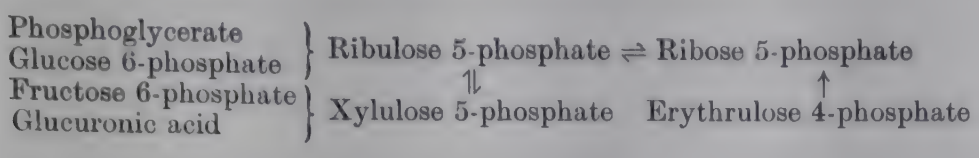
Except in a few rare instances, the sugar concerned is either D-ribose or 2-deoxy-D-ribose (R_1 = OH or H), present invariably in the furanose form. Again, only very few exceptions are known to the rule that the glycosidic centre possesses the β-configuration and is combined at N-9

of the purine ring or N-1 of the pyrimidine ring. Many enzymes show specificities connected with the nature of the purine or pyridimine residue, but these fall outside the scope of this discussion. Discounting partial degradation products of nucleotides, or polynucleotides, a phosphoryl residue is always found esterified with the hydroxyl group at C-5′ of the carbohydrate. This may be united to other simple or combined phosphoryl residues as in the nucleotide coenzymes, in which case a phosphoric anhydride linkage is present, or it may be esterified with another nucleotide as in the polynucleotides. Finally, one of the hydroxyl groups on an asymmetric carbon atom of the sugar may carry a simple or a combined phosphoryl residue.

SYNTHESIS OF D-RIBOSE AND 2-DEOXY-D-RIBOSE

It is proposed first to examine the reactions leading to the synthesis of D-ribose in order to decide at what point or points in the sequence steric specificity arises. It seems likely that the principal route to ribose involves reactions of the pentose phosphate cycle. Thus glucose 6-phosphate may be converted via 6-phosphogluconate into D-ribulose 5-phosphate, which is then isomerized to D-ribose 5-phosphate. This route would result in the formation of the compound labelled at C-1 from glucose labelled at C-2. This labelling pattern is not always observed, however, and it is probable that other reaction sequences operate either as alternatives to the above, or simultaneously with it (Shreeve, 1959). Thus sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate may be converted by transketolase into D-ribose 5-phosphate and D-xylulose 5-phosphate. This latter compound is formed more directly from fructose 6-phosphate by transketolation with glyceraldehyde 3-phosphate and is converted by the enzyme phosphoribulose epimerase into ribulose 5-phosphate and thence to ribose 5-phosphate. Another possible route to ribose may exist with D-glucuronic acid as starting material, but since this also would involve D-xylulose as an intermediate, this route does not need special consideration for the present purpose (Eisenberg, Dayton & Burns, 1959). Other possibilities exist, including, for example, the condensation of formaldehyde with D-erythrulose 1-phosphate (Charalampous, 1955), but it is probable that the generally important routes to ribose involve D-xylulose 5-phosphate and D-ribulose 5-phosphate as in the reaction sequences discussed above. Furthermore, in photosynthetic organisms, reactions of the pentose phosphate cycle play a role also in the regeneration of ribulose 5-phosphate from phosphoglycerate. To summarize, it may be said that the formation of D-ribose 5-phosphate

largely depends on the stereospecificity of one or both of the two enzymes phosphoribuloepimerase and phosphoribose isomerase:



These two enzymes are present in a variety of tissues, and a detailed study has been made of the isomerase and epimerase of animal tissues by Dickens & Williamson (1956). They found that the position of equilibrium for the epimerase reaction slightly favours the formation of xylulose 5-phosphate, so that the formation of ribose 5-phosphate is not kinetically controlled by the epimerization and will therefore be determined more by the isomerase reaction.

The phosphoribose isomerase described by Dickens & Williamson has many properties in common with enzymes from other sources (Axelrod & Jang, 1954; Srere, Cooper, Tabachnick & Racker, 1958; Horecker, Smyrniotis & Seegmiller, 1951), the position of equilibrium apparently varying slightly from one enzyme to another. These results are subject to some error, however, since it is difficult to ensure complete removal of the epimerase from the isomerase. The most pertinent finding for the present discussion is that the isomerase of animal tissues shows a high degree of specificity when tested with a number of pentoses and pentose phosphates. No conversion is observed with D-xylose 5-phosphate and D-arabinose 5-phosphate, nor with the free pentoses D-ribose, D-xylose and D-arabinose. Furthermore, the enzyme is inhibited by 5-phosphoribonic acid. Thus the key reaction in the formation of the ribose structure is the final one of the sequence. How this specificity arises cannot yet be stated, but it appears that phosphoribose isomerase has a functional thiol group since, although it is not affected by iodoacetate, mercurial compounds produce an inhibition which is reversed by cysteine. The nature of the enzyme–substrate complex remains to be investigated, but it is tempting to suggest that three-point attachment to the intermediate enediol may be involved. Finally, it may be mentioned that, since other pentose phosphates commonly arise in tissues, the almost universal occurrence of ribose in the pentose nucleotides must depend on specificities of enzymes concerned in later stages in their synthesis. It is important to note that the reaction sequences discussed above lead, not to the free sugar but to ribose 5-phosphate, subsequent reaction of which ensures the maintenance of the furanose structure in the nucleotides produced.

Analysis of the reactions leading to derivatives of 2-deoxyribose is not likely to give information on the occurrence of stereospecific reactions. A number of pathways have been suggested, but too little

information is at hand from experiments with purified enzymes to make their discussion profitable. Deoxyribonucleosides or deoxyribonucleotides are believed to be formed from ribose derivatives without fission of the glycosidic linkage and in *Escherichia coli*, ribose and deoxyribose are believed to have a common precursor (Bagatell, Wright & Sable, 1959). Since this implies a reduction of stereochemical complexity it does not come under consideration in the present context.

FORMATION AND FISSION OF GLYCOSIDIC BONDS

Nucleoside phosphorylases

With few exceptions, nucleotides possess a β-glycosidic linkage. Enzymes which cleave this bond have been known for very many years, but it was not until 1945 that it was shown (Kalckar, 1945) that the

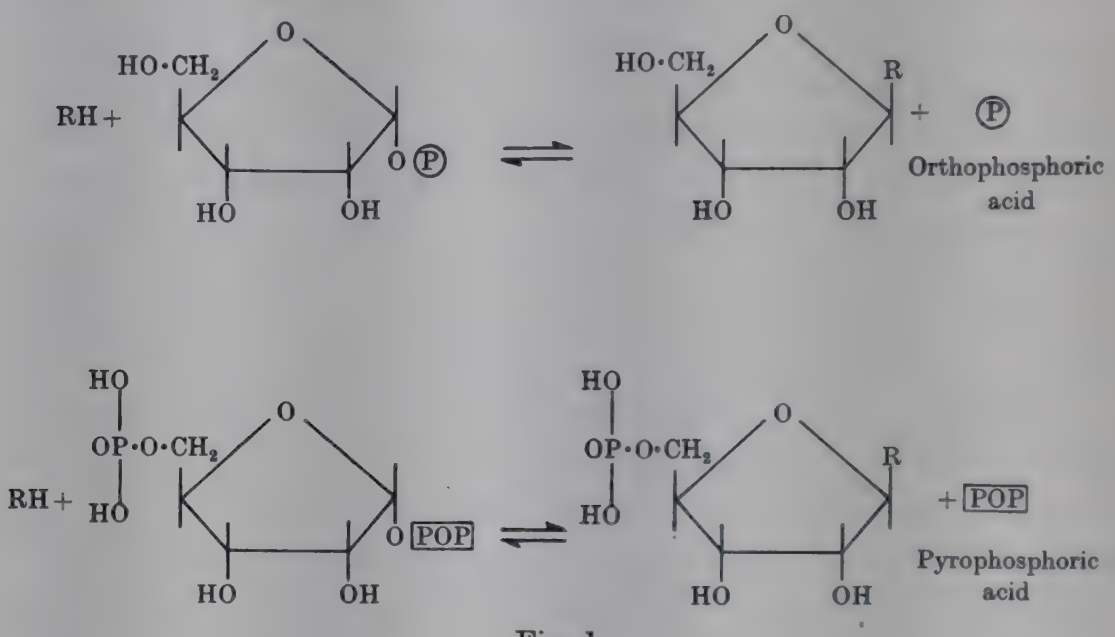
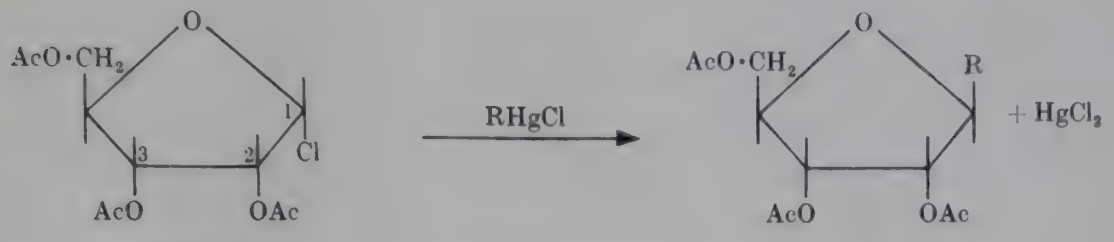


Fig. 1.

reaction consisted of a phosphorolysis rather than hydrolysis. True nucleoside hydrolases have since been found, but it is now realized that enzymes breaking the nucleosidic linkage fall into two main classes, namely the nucleoside phosphorylases and the nucleotide pyrophosphorylases. Fig. 1 shows typical reactions.

A number of enzymes catalysing these types of reaction are known, their specificities varying slightly, particularly as regards the nature of the base involved. They are all highly specific, however, for the same carbohydrate, namely α-D-ribofuranose 1-phosphate or 5-phospho-α-D-ribofuranose 1-pyrophosphate as the case may be. Most published work concerns nucleoside phosphorylase, but it may be seen that both types of reaction involve inversion of configuration at C-1 of the ribosyl

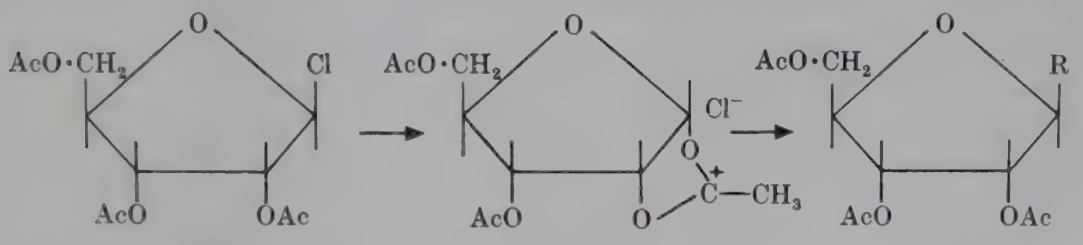
residue. The mechanism of the reaction is not known, but it is instructive to compare this reaction with analogous chemical syntheses of nucleosides. Many syntheses of the general type (III → IV) have been carried out with different halogenoacetyl sugars and the generalization has been made that the incoming purine ion will, for steric reasons, approach from the direction *trans* to the residue at C-2 irrespective of the original configuration at C-1 (Baker, Joseph, Schaub & Williams, 1954). This may take place by simple inversion when the halogen and the C-2 *O*-acetyl groups are *cis*. When these groups are in the *trans* disposition, a double



(III) (IV)

inversion may take place, either by initial inversion of the halogen by attack by halide ion or by participation of the acyl residue at C-2 (V–VII). Thus stereospecificity is exhibited in this type of non-enzymic reaction leading to the formation of the nucleosidic bond.

The nucleoside phosphorylases convert α-D-ribofuranose 1-phosphate into a β-nucleoside, and it is of interest to consider to what extent this



(V) (VI) (VII)

specificity is attributable to the enzyme and to what extent it is inherent in the nature of the reaction catalysed. If the mechanisms of the chemical and the enzymic reactions are in fact analogous, then the formation of a β-nucleoside is to be expected, but such an analogy cannot explain the requirement of the α-configuration of the ribose 1-phosphate. If the specificity did not arise at least in part from the nature of the enzyme, α-D-xylofuranose 1-phosphate would be expected to be a substrate, which is not the case (Fox, Codington, Yung, Kaplan & Lampen, 1958). Furthermore, it might be expected that nucleoside phosphorylases acting on deoxyribonucleosides would show a lower degree of specificity by analogy with the formation of both α- and β-nucleosides from 2-deoxysugars by chemical means (Cleaver, Foster &

Overend, 1959). It thus appears that the formation of a β-nucleoside may be attributable to the nature of the chemical reaction catalysed, but that the α-configuration of the ribosyl phosphate is a requirement of the enzyme itself.

In order to construct a working hypothesis concerning the way in which the enzyme exerts its effect, it is necessary to consider in more detail the mechanism of the analogous chemical reactions. Although the reaction (III $\rightarrow$ IV) may take place with simple inversion in a bimolecular process, a number of reactions at the anomeric position proceed by a unimolecular ionization to give a cation in which C-1 is trigonal (Lemieux & Shyluk, 1955). Substitution at C-1 is then directed by the shielding effect either of the withdrawing ion or of neighbouring groups (for the present purpose it is unnecessary to distinguish between these two possibilities). If a bimolecular mechanism operates, the view may be taken that the α-configuration of the phosphate is required because an adjacent hydroxyl group is not likely to participate in the reaction (Lemieux, 1954). If a unimolecular mechanism holds good, the question arises whether a solvated hydroxyl group at C-2 is sufficient to shield C-1 from attack from the α-direction. Even if this were the case, it would not explain why D-xylofuranose 1-phosphate is not a substrate. The conclusion is therefore reached that the hydroxyl groups at C-2 and C-3 are concerned in the formation of the enzyme–substrate complex. It is suggested that this is facilitated by combination of the enzyme with the α-phosphoryl residue, which may or may not remain attached to the enzyme after fission of the C-1 bond. If this is so, the incoming ion will inevitably take up the β-position (Fig. 2).

One further point deserves consideration. The reaction of α-D-ribofuranose 1-phosphate with purine or pyrimidine bases differs from similar chemical replacement reactions in being freely reversible. The reverse reaction, namely the phosphorolysis of a nucleoside, involves the approach of the bulky phosphoryl residue to C-1 in a position *cis* to the group at C-2, and steric resistance to this approach might well be encountered. This may account for the position of equilibrium lying well over to the nucleoside formation (Kalckar, 1945).

Nucleotide pyrophosphorylases

Comparatively little has been recorded about nucleotide pyrophosphorylases which catalyse reactions of 5-phosphoribosyl pyrophosphate, but it is possible that these reactions take place by mechanisms similar to those discussed above for nucleoside phosphorylases. Pyrophosphorylases are known which act on purine and pyrimidine nucleotides, and it appears that the reversal of the pyrophosphorolysis of orotidylic acid is a key reaction in the synthesis of pyrimidine nucleo-

tides *de novo*. Nevertheless, the enzyme catalysing the pyrophosphorolysis or orotidylic acid differs from other similar enzymes in favouring pyrophosphorolysis rather than nucleotide synthesis (Kornberg, 1957). The purine nucleotide pyrophosphorylases are probably not concerned principally with *de novo* synthesis of nucleotides, since the main synthetic pathway is probably that established by Buchanan and co-workers for pigeon liver (see Mehler, 1957). Whereas the formation of

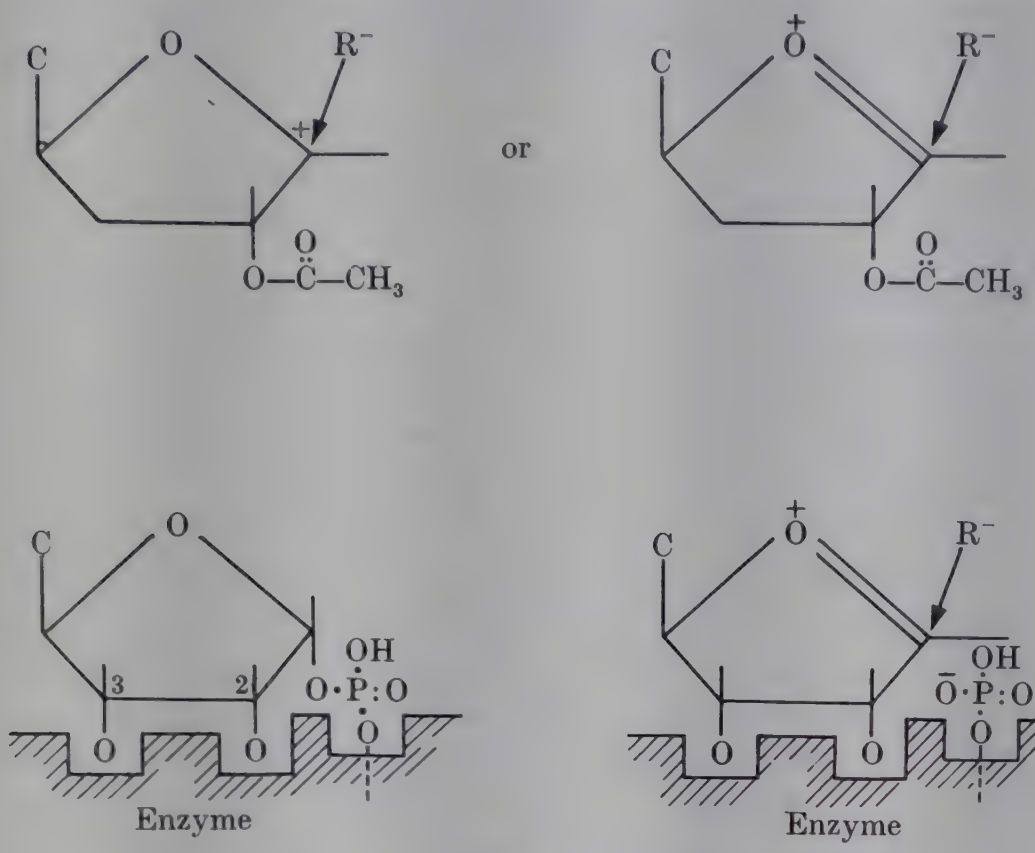


Fig. 2.

the glycosidic linkage at N-1 of the pyrimidines or N-9 of the purines by reversal of the phosphorylase or pyrophosphorylase reactions is dependent on the properties of the enzyme–substrate systems themselves, the formation of a 9-ribosylopurine by Buchanan's route is determined entirely by the sequence of reactions involved. The only reaction in which stereochemical selectivity plays a part is that in which 5-phosphoribosylamine is formed, and this is likely to be closely related to the pyrophosphorolyses discussed previously (cf. Wyngaarden & Ashton, 1959).

It is desirable to refer briefly to enzymes, principally of bacterial origin, which catalyse exchange of purine or pyrimidine bases with ribonucleosides or deoxyribonucleosides without the participation of phosphate. In these reactions, the β-configuration of the glycosidic bond is maintained. Here again, it is of interest to attempt to apportion responsibility for the steric course of the reaction between the enzyme

and the nature of the reaction which it catalyses. In this case, it is difficult to envisage a simple bimolecular inversion. Two possibilities may be suggested: first, that the initial process is a unimolecular dissolution of the glycosidic bond, the direction of entry of the new base being determined by the shielding effect of the enzyme; alternatively, the enzyme may act in a manner analogous to a participating group at C-2 by assisting the ejection of the base. In either case, it would be necessary to postulate that the enzyme is closely associated with the hydroxyl groups at C-2 and C-3, as in the phosphorylases.

FORMATION AND FISSION OF BONDS IN POLYNUCLEOTIDES

It has been established that the principal reactions leading to the formation of polynucleotides are the polymerization of nucleoside 5′-di- or -tri-phosphates catalysed by enzymes such as polynucleotide phosphorylase. These enzymes, using as substrates compounds commonly present in the acid-soluble fraction of tissues, are most likely to be responsible for net synthesis of polynucleotides. It has been demonstrated, however, that internucleotide linkages can be formed *in vitro* by pancreatic ribonuclease (Heppel & Whitfield, 1955; Barker & Parsons, 1955) and the possibility exists that the ribonucleases are concerned with breakdown and formation of polynucleotides under conditions where turnover rather than net synthesis occurs. As circumstantial evidence in favour of this, we have found that the activity of ribonuclease in cultures of *Micrococcus lysodeikticus* rises to a maximum at the onset of stationary phase, but is relatively lower in logarithmic phase (Barker & Cannon, 1960). Similar results have been observed by Nishimura & Nomura (1959), but their results are of doubtful significance since they were measuring what they believed to be an extracellular enzyme. However, it is pertinent to examine closely the synthetic as well as the degradative reactions of ribonuclease.

Pancreatic ribonuclease, which has been studied most, is known to degrade ribopolynucleotides by initial formation of pyrimidine nucleoside 2′:3′-hydrogen phosphates which are subsequently hydrolysed to nucleoside 3′-dihydrogen phosphates (Markham & Smith, 1952, Brown & Todd, 1953; Brown, Magrath & Todd, 1952). The enzymic hydrolysis of the cyclic phosphates, in which a steric factor is concerned, is analogous to the synthetic reaction catalysed by the enzyme, in which a pyrimidine nucleoside cyclic phosphate reacts with a hydroxylic compound to form a diester of phosphoric acid. In both cases, the 3′-phosphate alone is formed. These reactions of the cyclic phosphates in the presence of ribonuclease are similar to the non-enzymic hydrolysis

or to their reaction with alkoxide ions to yield alkyl esters of nucleotides (Barker, Montague, Moss & Parsons, 1957). With both chemical and enzymic catalysis, reaction takes place only with primary, but not with secondary, or tertiary, hydroxyl groups (Fig. 3). In contrast with the enzymic reaction, the product of the chemical catalytic reaction is not entirely the 3′-phosphate, but, on the other hand, steric factors are not entirely absent. As the size of the attacking ion increases from hydroxide through methoxide, ethoxide, *n*-propoxide, *n*-butoxide to benzyloxide, the proportion of the 3′-isomer formed increases from 60 to 80 %. This suggests that the enzymic reaction is the limiting case in a series such

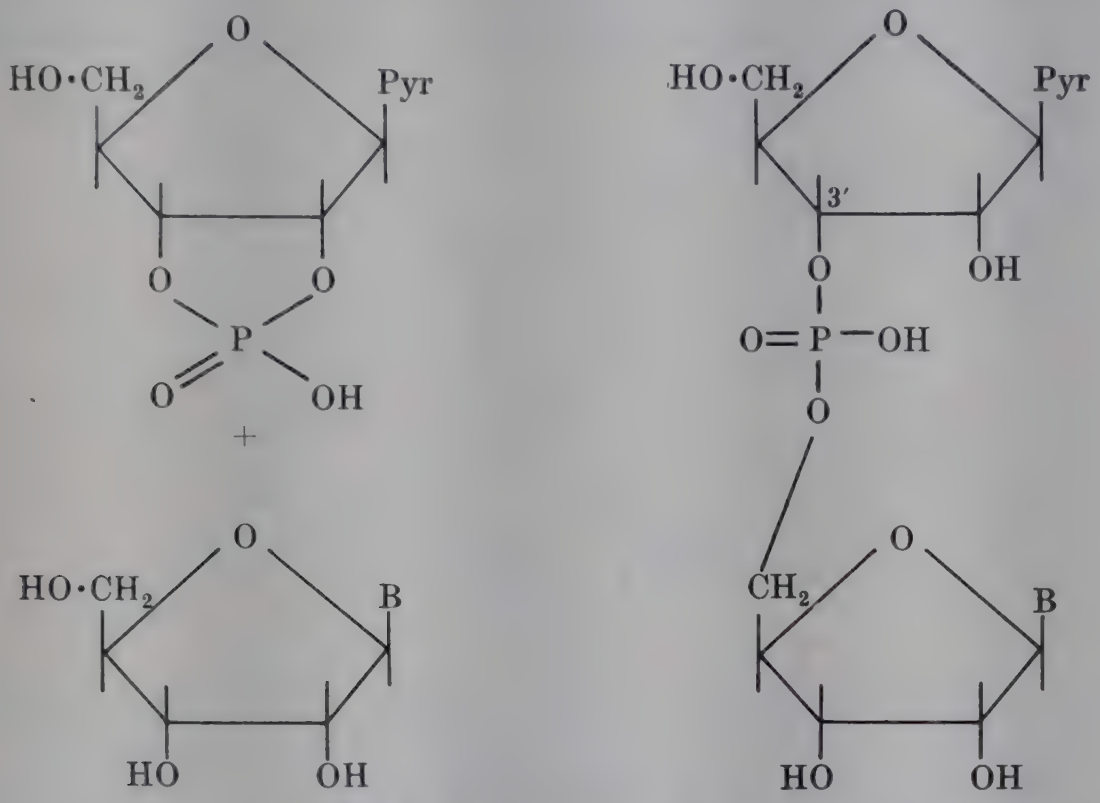
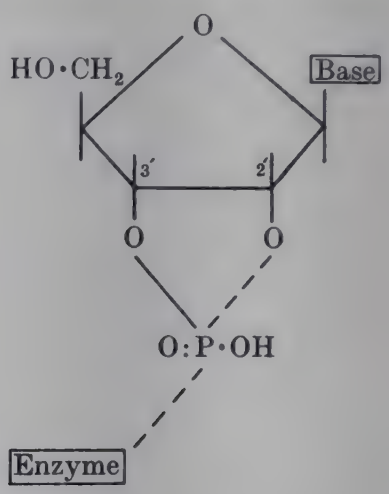


Fig. 3. Pyr, pyrimidine; B, base.

as this, and consists initially in the attack on the cyclic phosphate of a very large ion. Thus it appears that, in this case also, the steric effects observed in the enzymic reaction are due partly to the nature of the enzyme, but also partly to the nature of the reaction catalysed. This could be regarded as self-evident, since it is essential to treat the enzyme–substrate system as a whole, but it is frequently possible to put forward tentative proposals concerning enzyme–substrate complexes by considering possible ways in which observed steric effects could arise. Thus, in the alcoholysis of a nucleoside cyclic phosphate, attack by a large ion may have to come from a direction furthest from the large pyrimidine residue, which would result in fission of the bond between the phosphorus atom and the oxygen atom on C-2′, as suggested in formula (VIII). Further work is necessary to confirm this hypothesis.

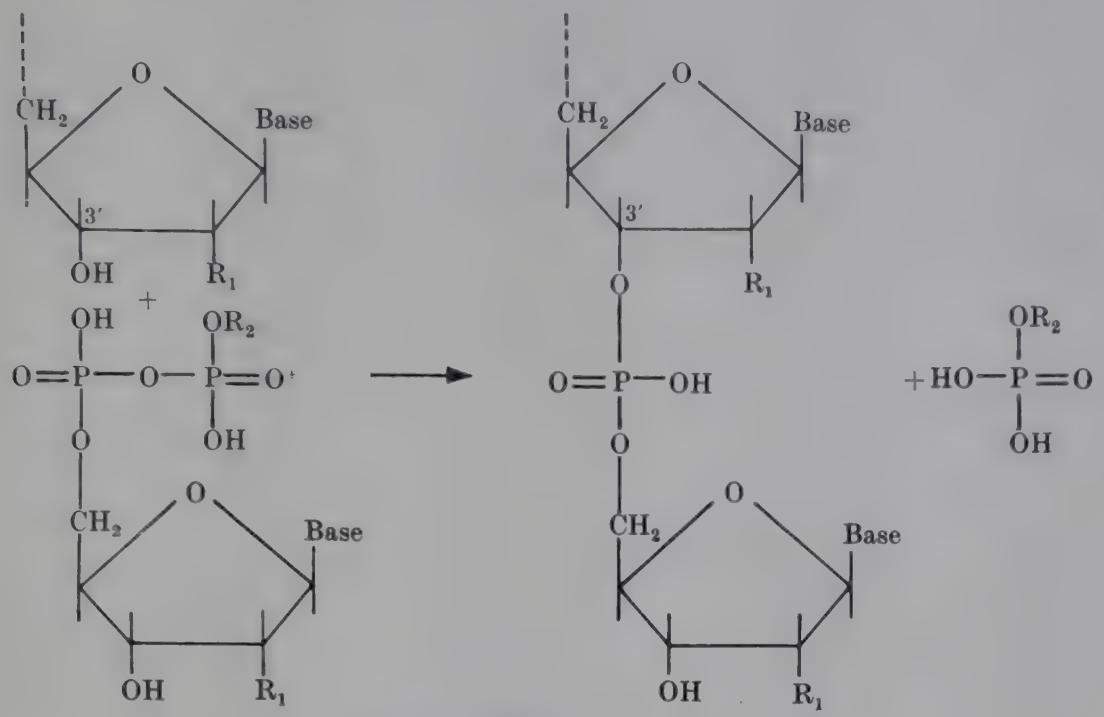
Finally, steric effects in the polynucleotide phosphorylases and related enzymes may be examined. The first enzyme of this type was described by Ochoa and co-workers (Grunberg-Monago, Ortiz & Ochoa, 1956) and brings about the polymerization of nucleoside 5′-pyrophosphates to polynucleotides, with the elimination of orthophosphate. As may be seen (Fig. 4), the reaction is a phosphorylation by a mixed anhydride, which differs from the more usual type in that it is the nucleotide half of the anhydride molecule which is esterified, yielding a diester of orthophosphoric acid. This aspect of the reaction does not enter into the present discussion and it is desired merely to point out that esterification occurs only at C-3′, since it has been shown that the polymers formed resemble natural polynucleotides in general structure. Nothing



(VIII)

can be said as yet about this selective attack at C-3′. The reaction is more complicated than Fig. 4 would suggest since, for instance, guanosine 5′-pyrophosphate is not polymerized unless other substrates are also present, such as adenosine 5′-pyrophosphate, and a polynucleotide 'primer' is also necessary. This type of effect is even more marked in the case of enzymes which form deoxyribopolynucleotides. These enzymes require the 5′-triphosphates and eliminate pyrophosphate (some enzymes acting in the ribose series also require triphosphates). All the nucleotides normally found in the natural polymer (RNA or DNA) are necessary for reaction to occur and in this case also a 'primer' is required (Kornberg, 1957). This reaction has been investigated in detail by Kornberg and his collaborators (Lehman *et al.* 1958) and it has been shown that the base composition of the polymer formed is governed by that of the 'primer'. This is a direct demonstration that the primer acts as a template and will no doubt be found in due course to be a most remarkable example of a stereochemical effect at the macromolecular level, in which the steric complexity of the product of the reaction is comparable with that of the enzyme itself.

Steric effects are present in many, if not all, enzymic reactions. To attribute the origin of the specificity necessarily to the properties and structure of the enzyme alone is to ignore the essential complementary nature of enzyme and substrate, and to miss the opportunity which stereochemical studies afford of gaining a closer understanding of enzymic processes.



R_1 = H or OH
R_2 = H or PO_3H_2

Fig. 4.

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CONCLUDING REMARKS

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I could not do justice to the excellence of the contributions to this Symposium if I attempted to summarize or present an overall picture. I shall therefore confine my remarks to a few personal impressions of this fascinating subject and of the stimulating discussion we have had today.

As one who has worked most of his life on the frontiers between chemistry and biology, I feel that I am almost a biochemist. I can at least appreciate the intricacy of the problems the biochemist has to face. Surely, the greatest of these problems is the nature of enzymic catalysis, but is it not also the most baffling? Are we much nearer to understanding it than when Fischer propounded his famous lock-and-key hypothesis? True, we have each in our little part of the biochemical mansion examined and measured many locks and often found the appropriate keys. But do we know in any one instance just *how* a given key unlocks a particular door? There is another thing. Our work is, of necessity, so specialized that a meeting such as this today gives us all a welcome opportunity to stand back a little and try to glimpse the relationship of our work to that of others in the same field, and to see where we are going. It clears the air, removes misconceptions and gives us new angles of approach. I am impressed by the wealth of knowledge which is piling up every day about the chemical mechanisms of living matter—the fine structure of substrates, the dissection of an overall chemical transformation into its constituent parts, the role of coenzymes, accelerators, inhibitors and the like—but there is one aspect which has always troubled me. It is the astonishing prodigality of Nature in the provision of biocatalysts so highly specific that we might almost say 'one reaction—one enzyme'. I have difficulty in believing that Nature is so uneconomic, despite the abundance of authenticated evidence for it. It becomes more bewildering still when one thinks that any given enzyme can be brought into being only by the agency of other enzymes, which have in their turn to be synthesized, and so, presumably *ad infinitum*. Perhaps the time is ripe for an all-out attack on the fine structure of an enzymic protein? I should like to know, for instance, what are the precise differences of physical and chemical structure of crystalline α-amylase and crystalline β-amylase which would account for the curious differences in their modes of action on starch.

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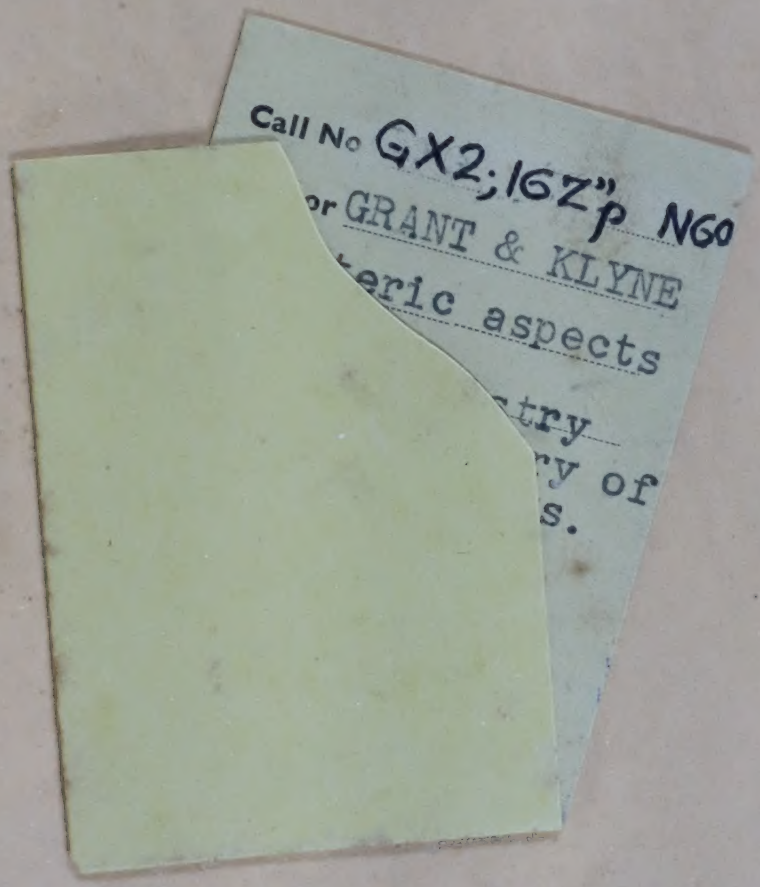
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